CROSSTALK BETWEEN STEROID AND GnRH RECEPTORS IN REGULATING GENE EXPRESSION IN THE MOUSE LβT2 GONADOTROPE CELL LINE

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own, original work, unless acknowledged otherwise. I hereby declare that I have not previously submitted any part of my work at any other university for this intended degree.

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Signature

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Date

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“Live as if you were to die tomorrow. Learn as if you were to live forever.” Mahatma Gandhi.

ABSTRACT

Sex hormones form the gonads and stress hormones from the adrenals are responsible for maintaining sexual development and regulating cellular responses to stress, respectively. It is becoming evident that endocrine signalling networks do not exist in isolation. Instead they are integrated, allowing the cell to respond to multiple signals from the environment. However, most studies addressing the effects of hypothalamic pituitary gonadal (HPG) and hypothalamic pituitary adrenal (HPA) hormonal feedback to the pituitary have been limited to the isolated actions of individual hormones. In this study we focus on the interplay of adrenal and gonadal hormone feedback regulating target receptor mRNA and protein expression in a pituitary gonadoptrope cell line, and discuss how crosstalk signalling may contribute to differential gene expression. The transcriptional effects of progesterone (P4), and estrogen (E2) from the gonads, and cortisol from the adrenals, and gonadotropin-releasing hormone (GnRH) from the hypothalamus, are mediated by the progesterone receptor (PR), estrogen receptor (ER), glucocorticoid receptor (GR) and gonadotropin-releasing hormone receptor (GnRHR), respectively. Therefore, the aim of this study was to investigate the effect of P4, E2, the synthetic GR agonist dexamethasone (Dex), and gonadotropin-releasing hormone (GnRH), on ER, PR, GR and GnRHR levels in gonadotrope cells. Combinations of some hormones were also used in an attempt to simulate the interplay of signalling events present under physiological conditions. The LβT2 mouse pituitary precursor gonadotrope cell line was used as a model system. Cells were initially characterised for basal steroid receptor (SR) mRNA and protein expression using conventional PCR and western blot techniques. Results show expression of ERα, PR (A+B) and GRα mRNA, and GRα protein. PR (A+B) protein expression was not detected using western blot techniques, while no conclusive results were obtained for ERα protein expression. Reporter assays reveal GR-mediated transactivation in response to agonist treatment, but no PR- or ER-mediated transactivation in response to agonist. Quantitative real-time PCR analysis provided strong evidence that receptor mRNA is ligand-dependently regulated in response to Dex, P4 and GnRH. Specifically, Dex was shown to regulate GnRHR, ERα and GRα mRNA levels; P4 was shown to regulate ERα and GRα mRNA levels; and GnRH treatment was shown regulate GnRHR, ERα, PR (A+B) and GRα mRNA levels in the LβT2 cell line. Combination treatments of Dex + GnRH appeared to act additively in regulating GnRHR and GRα mRNA. E2 priming appeared to modulate the Dex responsiveness on target GnRHR, ERα and GRα genes, suggesting cross talk between the ER and GR signalling pathways. Furthermore, both E2 and GnRH priming were shown to modulate hormone response element (HRE)-reporter gene activity. Finally ligand-dependent effects of Dex and GnRH on GRα mRNA levels appeared to be consistent with changes in GRα protein levels. Interestingly GnRHR expression was shown to be highly up-regulated (9.5 fold) in response to Dex

and GnRH, suggesting GnRHR signalling is greatly influenced by HPA and HPG crosstalk. Furthermore, ER and GR expression were shown to be down-regulated in response to GnRH, Dex and P4, suggesting that hypothalamic, adrenal and gonadal feedback, respectively, all play an important role on relative LβT2 steroid receptor levels. Whether these hormonal effects are also exhibited *in vivo* in primary gonadotrope cells remains to be determined. Although no mechanisms were established; the study does lay a platform for further research into novel HPG and HPA crosstalk mechanisms regulating endogenous GnRHR, ER, PR and GR mRNA and protein levels.

TABLE OF CONTENTS

LIST OF ABREVIATIONS

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AP-1	Activator protein-1
AF 1	Activation function 1
AF 2	Activation function 2
AF 3	Activation function 3
bp	Base-pair
BPE	Bovine pituitary extract
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
c-Fos	Cellular Fos
c-Jun	Cellular Jun
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
CRH	Corticotropin-releasing hormone
CTD	Carboxy terminal domain
CRE	cAMP response element
CREB	cAMP response element binding protein
DAG	Diacylglycerol
DBD	DNA binding domain
DEPC	Diethylpyrocarbonate
Dex	Dexamethasone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E2	17β-estradiol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNO	Endothelial nitric oxide
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor

ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FKBP51	FK506 binding protein 51
FKBP52	FK506 binding protein 52
FSH	Follicle-stimulating hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GRIP-1	Glucocorticoid receptor interacting protein-1
GnRH	Gonadotropin-releasing hormone
GnRHR	GnRH receptor
GPCR	G-protein coupled receptor
HAT	Histone acetyltransferase
hGR	Human GR
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HRE	Hormone response element
HRT	Hormone replacement therapy
HSP70	Heat-shock protein 70
HSP90	Heat-shock protein 90
IP3	Inositol-(1,4,5)-triphosphate
JNK	Jun N-terminal kinase
Kd	Equilibrium dissociation constant
LB	Luria-Bertani
LBD	Ligand binding domain
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
MMTV	Mouse mammalian tumor virus
MOPS	4-morpholine-propanesulfonic acid
mRNA	Messenger ribonucleic acid
NCoA3	Nuclear co-activator 3
NTD	Amino terminal domain
NF-κB	Nuclear factor-kappa B
P4	Progesterone

p300	Adenovirus E1A-binding protein 300
PR	Progesterone receptor
PRE	Progesterone response element
PBS	Phosphate-buffered saline
PKA	Protein kinase A
PKC	Protein kinase C
POMC	Pro-opiomelanicortin
R5020	Promegestone
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Room temperature
Ser-211	Serine 211
Ser-226	Serine 226
SDS	Sodium dodecyl sulfate
SR	Steroid receptor
SRC-1	Steroid receptor co-activator-1
SRC-2	Steroid receptor co-activator-2
TAE	Tris-acetate EDTA
TAT	Tyrosine aminotransferase
TBS	Tris-buffered saline
TE	Tris-EDTA
UTR	Untranslated region
v/v	Volume per volume
w/v	Weight per volume
β-gal	Beta-galactosidase

HYPOTHESES & STRATEGIES

The central hypothesis of this study is that hormone-specific receptor (GnRHR, ER, PR, and GR) mRNA and protein levels are regulated in response to hormone treatments in the LβT2 cell line.

To assess this hypothesis, a number of aims were formulated and strategies were explored.

a) HORMONE TREATMENTS INFLUENCE GnRHR mRNA EXPRESSION IN THE LβT2 CELL LINE

i) Effects of Dex and GnRH on GRE-mediated transcription

A previous study performed by Kotitschke *et al.,* (2009) has shown that Dex, GnRH and combinations thereof increase the transcriptional activity on a GRE promoter in the LβT2 cell line. Therefore, it is necessary to reproduce this published result in the current cell line before exploring novel signalling pathways.

This was tested by examining the effect of saturating Dex and GnRH (100 nM) and combination treatments thereof on GR-mediated transactivation on a TAT-GRE-luc reporter construct in the LβT2 cell line. Assuming the hypothesis, a ligand-dependent trend in GRE-reporter activity will be observed with hormone treatments.

ii) Effects of Dex and GnRH on endogenous GnRHR expression in the LβT2 cell line

Further results obtained by Kotitschke *et al.,* (2009) have shown that the effects of Dex and GnRH on GRE-luc reporter activity are also observed on endogenous *GnRHR* mRNA expression in the LβT2 cell line. Therefore, to determine whether the cell line is responding according to the literature, this result needed to be reproduced in the hands of the current author.

This was tested using real-time PCR and *GnRHR*-specific primer pairs to examine ligand-induced effects on endogenous *GnRHR* mRNA expression levels in the LβT2 cell line. Assuming the hypothesis, expression of endogenous *GnRHR* mRNA, relative to vehicle, will follow a reproducible ligand-dependent trend, in relation to treatment with Dex, GnRH and combinations thereof (Kotitschke *et al.,* 2009).

iii) Effects of E2 and P4 signalling on endogenous GnRHR expression in the LβT2 cell line

Considering the outcome of (ii), the LβT2 cell line was to be used to further explore novel signalling pathways regulating *GnRHR* mRNA levels.

E2 and P4 signalling has been shown to regulate a number of gonadotrope responses via the HPG signalling axis (Ng *et al.,* 2009; Sleiter *et al.,* 2009). Furthermore, recent publications further support GnRHR and PR/ER crosstalk-signalling (An *et al.,* 2009; Chen *et al.,* 2009) in regulating target gene expression in LβT2 cells. Therefore, it was hypothesised that ER- and PR-mediated signalling pathways would regulate *GnRHR* mRNA expression levels.

To assess this hypothesis, a similar approach was used as described in (ii). Relative expression levels of *GnRHR* mRNA in response to treatment with E2 (0.2 nM) and P4 (100 nM) and subsequent combinations thereof was investigated with real-time PCR.

b) SR- AND GnRHR-MEDIATED SIGNALLING INFLUENCES ERα, PR (A+B) AND GRα EXPRESSION AND FUNCTION IN THE LβT2 CELL LINE

i) SR- and GnRHR-mediated signalling influences ERα mRNA and protein expression levels

According to the literature ERα mRNA is down-regulated in reproductive tissues including breast cancer (Alexander *et al.,* 1990) and uterus cells (Hsueh *et al.,* 1990) in response to P4 treatments. These effects may be due to the presence of an AP-1 site (Tang *et al.,* 1997) and a half-PRE site (Amicis *et al.,* 2009) in the ERα promoter. Furthermore ligand-dependent responses on ERα expression levels have been shown to be mediated by the PR (Amicis *et al.,* 2009).

Therefore, the hypothesis is that ERα mRNA and protein levels are regulated in response to treatment with Dex, P4, GnRH, E2 and combinations thereof in the LβT2 cell line.

The strategy to test this hypothesis was to use real-time PCR and ERα-specific primer pairs to investigate the transcriptional effects of ligand treatment on endogenous *ERα* mRNA expression. Protein levels were assessed by using Western blotting and an ERα-specific antibody. Unless

otherwise stated, the ligands Dex, P4, E2 (0.2 nM) and GnRH and combinations thereof were used at saturating concentrations of 100 nM.

Assuming the hypothesis is true, a reproducible and ligand-dependent trend was anticipated with varying ERα mRNA and protein levels in the LβT2 cell line.

ii) SR- and GnRHR-mediated signalling influences PR (A+B) mRNA and protein expression levels

Studies have shown the PR-B and PR-A promoters are up-regulated in response to E2, even though no consensus palindromic estrogen response element (ERE) can be found in their respective promoters (Kastner *et al.,* 1990). In primary pituitary gonadotrope cells, PR mRNA expression is found to be up-regulated in response to prolonged treatments with 0.2 nM E2 (Turgeon and Waring, 2006). Furthermore, in rat pituitary cells PR mRNA is up-regulated in response to E2, and down-regulated in response to progesterone (Turgeon and Waring, 2000).

Therefore the hypothesis is that PR (A+B) mRNA and protein levels are regulated in response to treatment with Dex, P4, GnRH, E2 and combinations thereof in the LβT2 cell line.

The strategy to test this hypothesis was to use real-time PCR and PR-(A+B)-specific primer pairs to study the transcriptional effects of ligand treatment on endogenous *PR-B* mRNA expression. Protein levels were assessed by western blotting with PR-B-specific antibodies.

Assuming the hypothesis is true, a reproducible and ligand-dependent trend will be observed in response to differential PR-B mRNA and protein expression levels in the LβT2 cell line.

iii) SR- and GnRHR-mediated signalling influences GRα mRNA and protein expression levels

In the literature GR mRNA and protein is down-regulated in response to glucocorticoids (Burnstein *et al.,* 1990; De Silva *et al.,* 1993). These transcriptional effects may be mediated by a half-GRE or a *cis*-element resembling an NF-κB site in the GRα promoter region (Breslin *et al.,* 2001)

Therefore the hypothesis is that GRα mRNA and protein levels are regulated in response to treatment with Dex, P4, GnRH, E2 and combinations thereof in the LβT2 cell line.

The strategy to test this hypothesis was to use real-time PCR and GRα-specific primer pairs to examine the transcriptional effects of ligand treatment on endogenous *GRα* mRNA expression. Protein levels were assessed by using western blotting and a GRα-specific antibody.

Assuming the hypothesis is true, a reproducible and ligand-dependent trend will be observed with regard to varying GRα mRNA and protein levels in the LβT2 cell line.

c) THE EFFECT OF LIGAND-INDUCED RESPONSES ON HRE-luc EXPRESSION IN THE LβT2 CELL LINE.

A central question of this part of the study was to assess whether ligand-activated ER, PR and GR are functionally active and can regulate promoter activity in the LβT2 cell line.

This was tested by measuring SR-specific agonist transactivation on an HRE-luc-reporter construct. The functionality of SRs was assessed by monitoring HRE-luc activity in response to SR-specific agonist treatments, at saturating concentrations (100 nM). Differences in relative HRE-reporter activity between vehicle and treated LβT2 cells will confirm the hypothesis that ligand-activated SRs can modify HRE-reporter activity in LβT2 cells. Assuming the null hypothesis, no differences in HRE-reporter activity would be seen for ligand pre-treatments relative to control, suggesting ligand treatments do not modulate HRE-reporter activity.

CHAPTER 1

LITERATURE REVIEW

1.1 Hypothalamic pituitary signalling

The hypothalamus is a small region of the brain that is responsible for integrating both nervous and endocrine signalling systems. It interprets signals from the nervous system to regulate the pituitary. The pituitary gland controls the majority of several endocrine organs in the human body. This interaction between the hypothalamus, pituitary and other endocrine glands is known as the hypothalamic-pituitary-endocrine axis, which controls all endocrine activity.

The pituitary gland consists of two components, the posterior and the anterior pituitary. The posterior pituitary originates from neural tissue, containing neural axons from the hypothalamus. The anterior pituitary comprises of glandular cells, consisting of a heterogeneous mixture of cell types, each specialised in synthesising and secreting key peptide hormones involved in endocrine signalling (Figure 1.1).

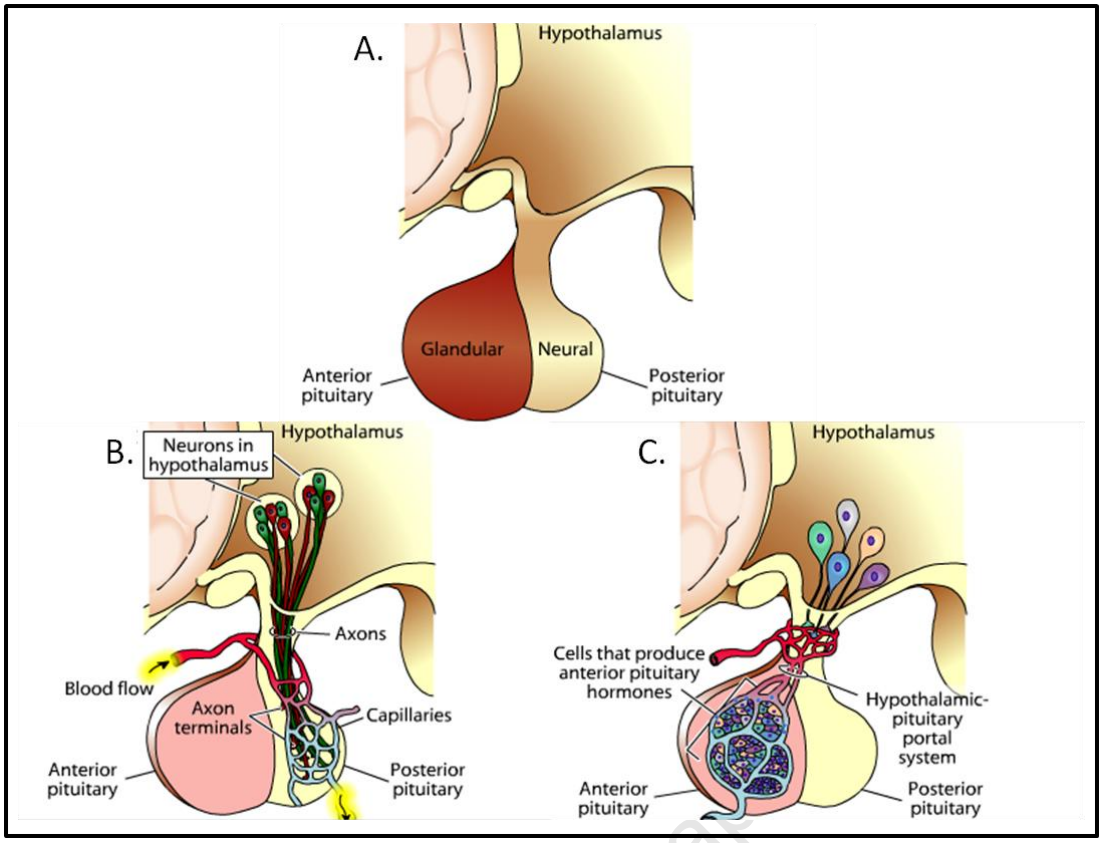

Figure 1.1 (A-C): The anatomy of the hypothalamus and pituitary. A: Representations of the cellular make up of the anterior and posterior pituitary. B: Schematic representation of the capillary network responsible for mediating the transportation of posterior pituitary hormones into the blood circulation. C: Schematic representation of the portal system mediating the transportation of hypothalamic hormones to their designated cell targets in the anterior pituitary [adapted from Purves (2004)].

1.1.1 Gonadotropes and gonadotrope cell lines

1.1.1.1 Cell types of the anterior pituitary

The anterior pituitary consists of a heterogenous population of differentiated endocrine cell-types, each responsible for the production of different hormones and the control of different endocrine signalling axes. The corticotropes differentiate first during pituitary development (Horn *et al.,* 1992) and produce a precursor peptide, pro-opiomelancortin (POMC), which is cleaved into several products including adrenocorticotrophin hormone

(ACTH), endorphin and enkephalin. Other cell types that differentiate later are the thyrotropes, which secrete thyroid-stimulating hormone (TSH), somatotropes, responsible for growth hormone production, lactotropes, which produce prolactin (PRL), and the gonadotropes, which produce the gonadotropin luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Horn *et al.,* 1992).

1.1.1.2 Gonadotrope cell lines

Within the anterior pituitary, gonadotropes make up 6-15% of the total cell population (Hyde *et al.,* 1982; Ibrahim *et al.,* 1986). In the past, experiments investigating the function of the gonadotrope's in reproduction have been performed *in vivo* or in primary pituitary cultures. However, the heterogeneity of the anterior pituitary cell population limits the interpretation of results derived from these studies. Furthermore, primary pituitary cells cannot be maintained in continuous culture, creating practical problems for experimental design. Therefore it was necessary to engineer immortalised pituitary cell lines, specifically gonadotrope cell-types, to use as model systems for the investigation of gonadotrope endocrine signalling. As a result, Dr. P. Mellon developed the αT3-1 and LβT2 cell lines and characterised them.

Numerous studies assessing GnRHR, gonadotropin signalling and gene expression have been carried out in the αT3-1 (Duval *et al.,* 1997a; Duval *et al.,* 1997b; White *et al.,* 1998; Norwitz *et al.,* 2002) and the LβT2 cell lines (Thackray *et al.,* 2009; An *et al.,* 2009; Chen *et al.,* 2009; Kotitschke *et al.,* 2009). All these studies have been shown to hold physiological relevance in interpreting molecular mechanisms of gene expression and regulation.

1.1.1.3 The αT3-1 cell line

The αT3-1 clonal cell line was created by targeted tumourigenesis in transgenic mice, using the promoter of the human glycoprotein hormone α-subunit (α-GSU) gene (Windle *et al.,* 1990). These cells represent precursor gonadotrope cells retaining some gonadotrope functions, including the expression of functional gonadotropin-releasing hormone receptor (GnRHR) and GnRHR-mediated responsiveness to gonadotropin-releasing hormone (GnRH) treatments. Additionally, they express several activin receptor subunits, as well as the β-subunits, but not the α-subunit of inhibin (Fernandez-Vazquez *et al.,* 1996). These cells also

express, synthesise and secrete the gonadotropin hormone α-subunit, but they do not express either of the gonadotropin hormone-specific β-subunits, LHβ and FSHβ (Windle *et al.,* 1990).

1.1.1.4 The LβT2 cell line

Like the αT3-1 cells, the LβT2 cell line was generated by targeted tumourigenesis. However, in this instance the rat LHβ promoter was used, generating a clonal cell line representing a more mature and differentiated gonadotrope cell (Turgeon *et al.,* 1996). These cells express functional GnRHR, the gonadotrophin hormone α-subunit and β-subunits LHβ and FSHβ (Turgeon *et al.,* 1996; Pernasetti *et al.,* 2001) and have been reported to express functional steroid receptors including the progesterone receptor (PR), estrogen receptor (ER) and glucocorticoid receptor (GR) (An *et al.,* 2009; Chen *et al.,* 2009; Kotitschke *et al.,* 2009). In addition, this cell line also expresses activin and activin receptors, as well as inhibin and follistatin, therefore displaying all the hallmarks of fully differentiated gonadotrope cells (Pernasetti *et al.,* 2001).

1.1.2 The hypothalamic-pituitary-gonadol axis (HPG)

The mammalian HPG axis controls reproduction, including sexual development, puberty, gametogenesis, pregnancy and menopause. The axis is governed by the pulsatile secretion and binding of the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) to the gonadotrope cell surface (Figure 1.2) (Levine and Ramirez, 1982). It activates the GnRH Receptor (GnRHR), a 328 amino acid G-protein coupled receptor, essential for maintaining serum gonadotropins (Burns and Matzuk, 2002). This is achieved through regulating the synthesis and release of the two heterodimeric glycoproteins gonadotropin hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH). Changes in the amplitude and frequency of GnRH pulses have differential effects on the rate on gonadotropin synthesis, resulting in differential secretion patterns of the gonadotropins, thereby regulating downstream hypothalamic pituitary gonadol signalling (Shupnik, 1996).

The gonadotropin hormones are composed of a common α subunit and specific β subunits, namely luteinizing hormone β (LHβ) and follicle stimulating hormone β (FSHβ) (Gharib *et al.,* 1990; Clayton & Catt, 1981; Pierce & Parsons, 1981). Absence of GnRH input to the pituitary

inhibits both gondadotropin hormone production and gonadal function in mammals (Mason *et al.,* 1986). Naturally occurring mutations in the GnRHR gene, resulting in GnRH resistance, have been found in patients with hypogonadotropic hypogoandism (HHG) (de Roux and Milgrom, 2001). Symptoms of HHG include delayed onset of puberty, absence of secondary sexual characteristics or low sex hormone levels (Seminara *et al.,* 1998; Millar *et al.,* 2004). Furthermore clinical studies have shown that the pulsitile administration of GnRH is able to induce ovulation and restore fertility in HHG woman (Seminara *et al.,* 2000). These studies highlight the importance of GnRHR-mediated signalling with regards to the regulation of reproduction, and reveal why GnRHR agonists and antagonists are widely used in the clinical treatment of infertility and hormone dependent diseases (Neill, 2002).

The pulsitile release of both LH and FSH is central to endocrine signalling (Figure 2), and regulation of follicular development, ovulation and steroidgenesis in females, and spermatogensis, testicular growth and steroidogenesis in males (Burns and Matzuk, 2002). Both are secreted into the blood stream from the pituitary and bind to their respective G-protein coupled receptors, FSH receptor (FSHR) and LH receptor (LHR), present in gonadal cells (Kumar *et al.,,* 1997).

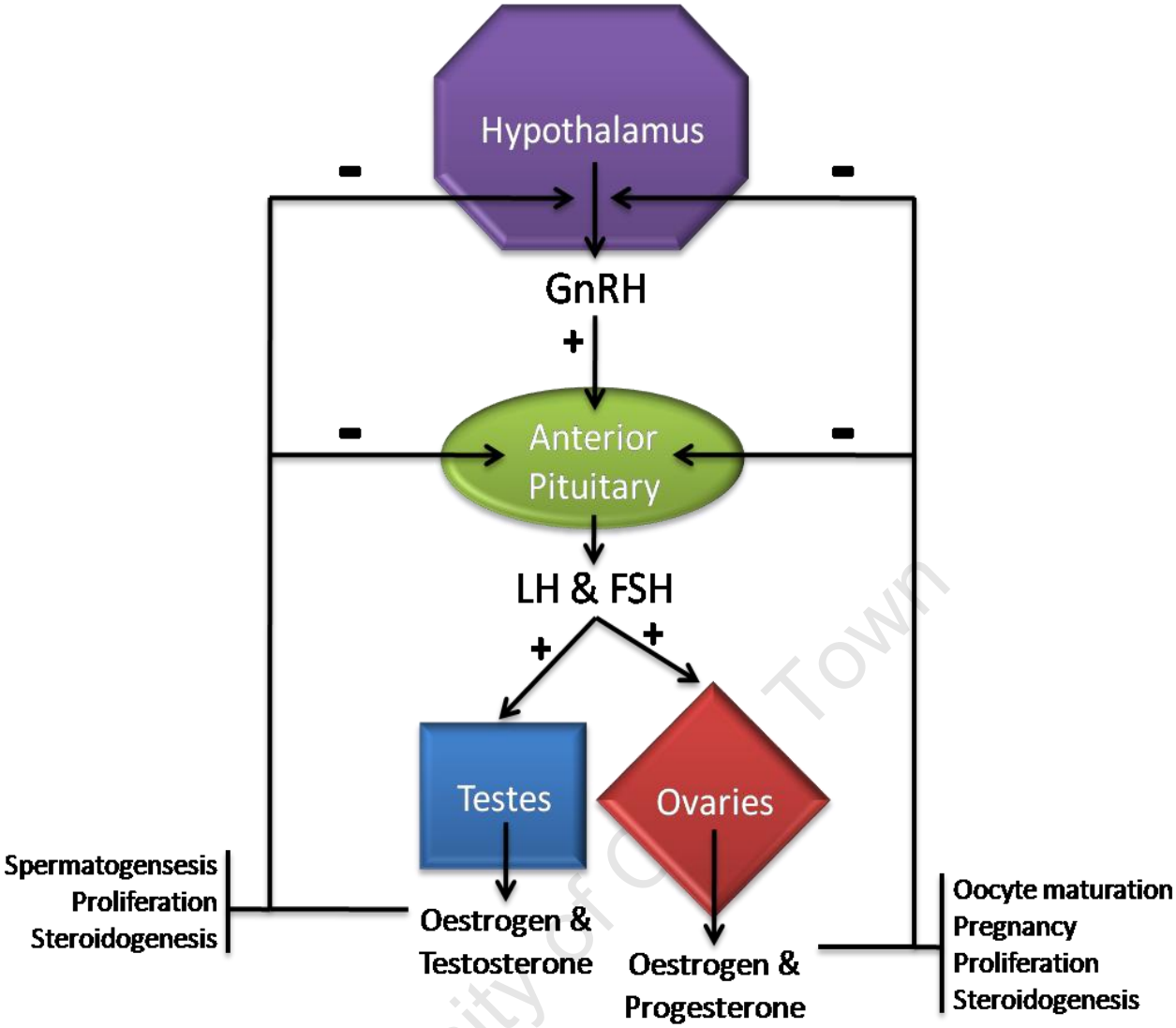

Figure 1.2: The HPG signalling axis. A model representing the major regulatory hormones and endocrine organs involved in HPG signalling. Arrows indicate the points of regulation mediated by hormones along the HPG axis. Arrows indicated positive (+) or negative (-) feedback effects of hormones. Abbreviations GnRH, LH and FSH are Gonadotropin-releasing hormone, Leuteinizing hormone and follicle stimulating hormone, respectively [figure adapted from Fernandes (2007)]

Gonadotrophins regulate the production of steroids by inducing the expression of gonadal steroidogenic enzymes responsible for the synthesis of various steroid hormones including testosterone, progesterone and estrogen (Burns and Matzuk, 2002). These steroid hormones then feedback on the hypothalamus and pituitary, in turn regulating *GnRH*, *LH* and FSH expression and secretion (Figure 2) (Ng *et al.,* 2009; Sleiter *et al.,* 2009; Burns &

Matzuk, 2002). This negative feedback is evidence for a complex signalling network that integrates and regulates HPG signalling.

Furthermore, the role of hormones and steroid receptors (SRs) in mediating negative feedback signalling is supported in transgenic mice studies, where alterations in SR activity or steroid biosynthesis result in problems with sexual function. Estrogen Receptor α (ERα) knockout mice exhibit female infertility that is associated with ovarian cyst formation (Schomberg *et al.,* 1999), as disruption of estrogen (E2) feedback signalling results in continuous gonadotropin subunit mRNA expression at the level of the pituitary (Scully *et al.,* 1997) and elevated LH levels in serum (Couse and Korach, 1999; Hess *et al.,* 1997). Additional studies on transgenic mice with abrogated E2 production reveal a female phenotype resembling that of ERα knockout mice, where high LH and FSH serum levels lead to infertility through ovarian cyst formation and inhibition of follicular development (Fisher *et al.,* 1998). The male phenotypes exhibit infertile characteristics brought about through an arrest in early spermatogenisis, germ cell apotosis, Leydig cell hyperplasia and high LH levels (Robertson *et al.,* 1999).

1.1.3 The hypothalamic pituitary adrenal axis (HPA)

The mammalian HPA axis is activated in response to stress, and regulates the synthesis of the endogenous glucocorticoid cortisol, secreted from the adrenal cortex. During a time of inflammation, lymphocytes/macrophages are activated. This leads to the production of inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). These cytokines induce the expression of pro-opiomelanicortin (POMC), the pre-cursor of corticotrophin-releasing hormone (CRH). CRH from the hypothalamus induces the synthesis and secretion of adrenocorticotropic-releasing hormone (ACTH) in corticotrope cells in the anterior pituitary. ACTH enters into the blood circulation system, and stimulates the adrenal cortex, resulting in the synthesis and secretion of glucocorticoids (Figure 1.3) (Smoak and Cidlowski, 2004). Glucocorticoids are anti-inflammatory hormones which affect various cell types including the hypothalamus, corticotropes, gonadotropes, T-cells, macrophages, eosinophils, neutrophils, mast cells, endothelial and epithelial cells (Kotitschke *et al.,* 2009; Smoak and Cidlowski, 2004).

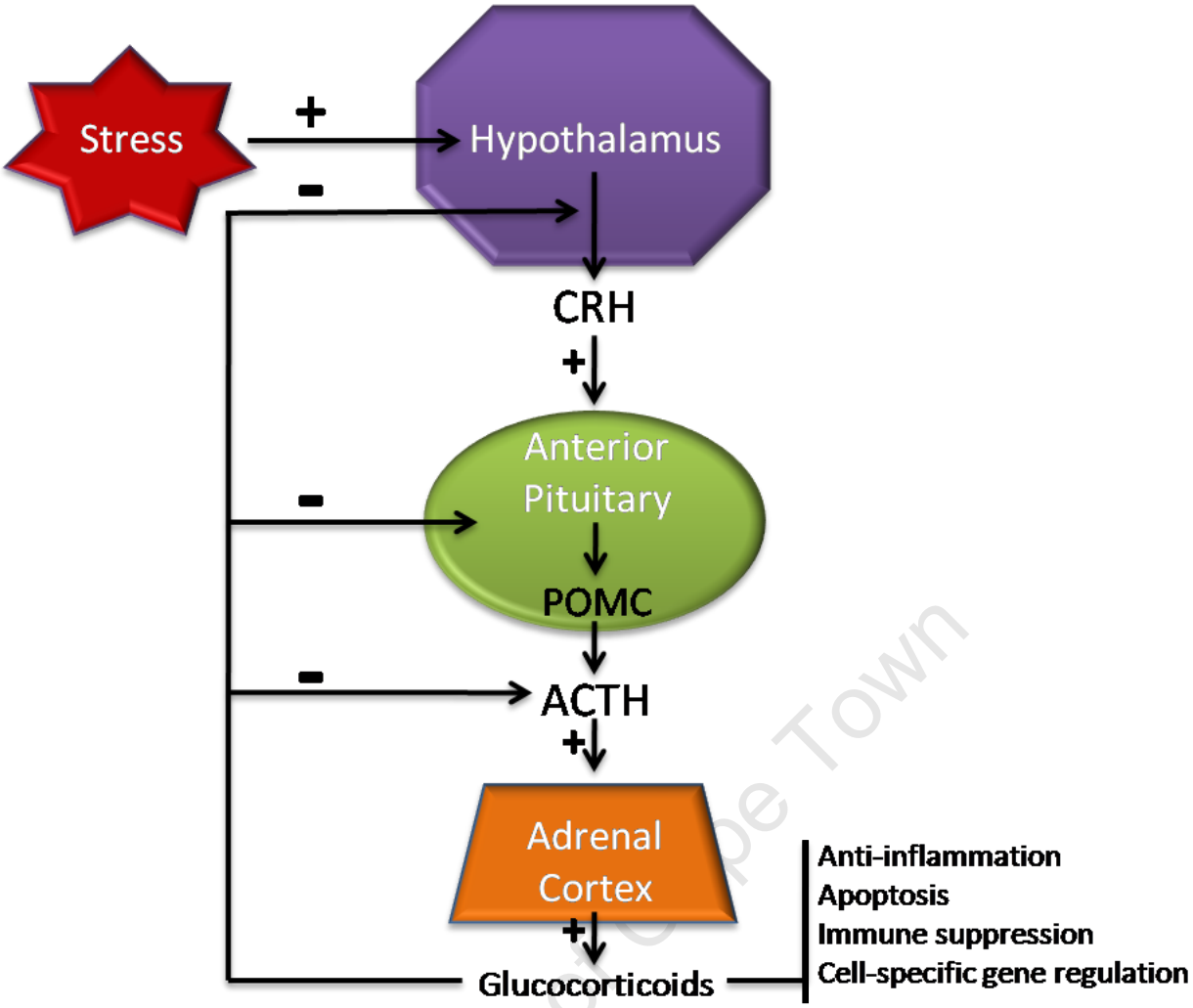

Figure 1.3: The HPA signalling axis. A model representing the major regulatory hormones and endocrine organs involved in HPA signalling. Arrows indicate the points of regulation mediated by hormones along the HPG axis. Arrows indicated positive (+) or negative (-) feedback effects of hormones. Abbreviations CRH, POMC and ACTH are corticotropin-releasing hormone, pro-opiomelanicortin and adrenocorticotropic hormone, respectively [figure adapted from Boetticher (2008)].

Glucocorticoids exert their anti-inflammatory effects through interrupting pro-inflammatory gene expression, cytokine-mediated signalling pathways and promoting apoptosis in certain immune cell types (Smoak and Cidlowski, 2004). Glucocorticoids are internally regulated through a negative feedback signalling mechanism (Figure 1.3) (Newton, 2000).

1.1.4 HPG-HPA cross-talk

In times of stress, the sympathetic nervous system and HPA signalling allows an organism to mobilize all of the body's resourses. Crosstalk signalling between HPG and HPA endocrine signalling pathways has been shown to modulate *GnRH* expression in the hypothalamus (Ferin, 1999; Berga *et al.,* 1989; Chandran *et al.,* 1994; Dubey and Plant, 1985), and has been associated with a reduction in LH secretion from the anterior pituitary (Figure 1.4) (Berga *et al.,* 1989; Baldwin *et al.,* 1991).

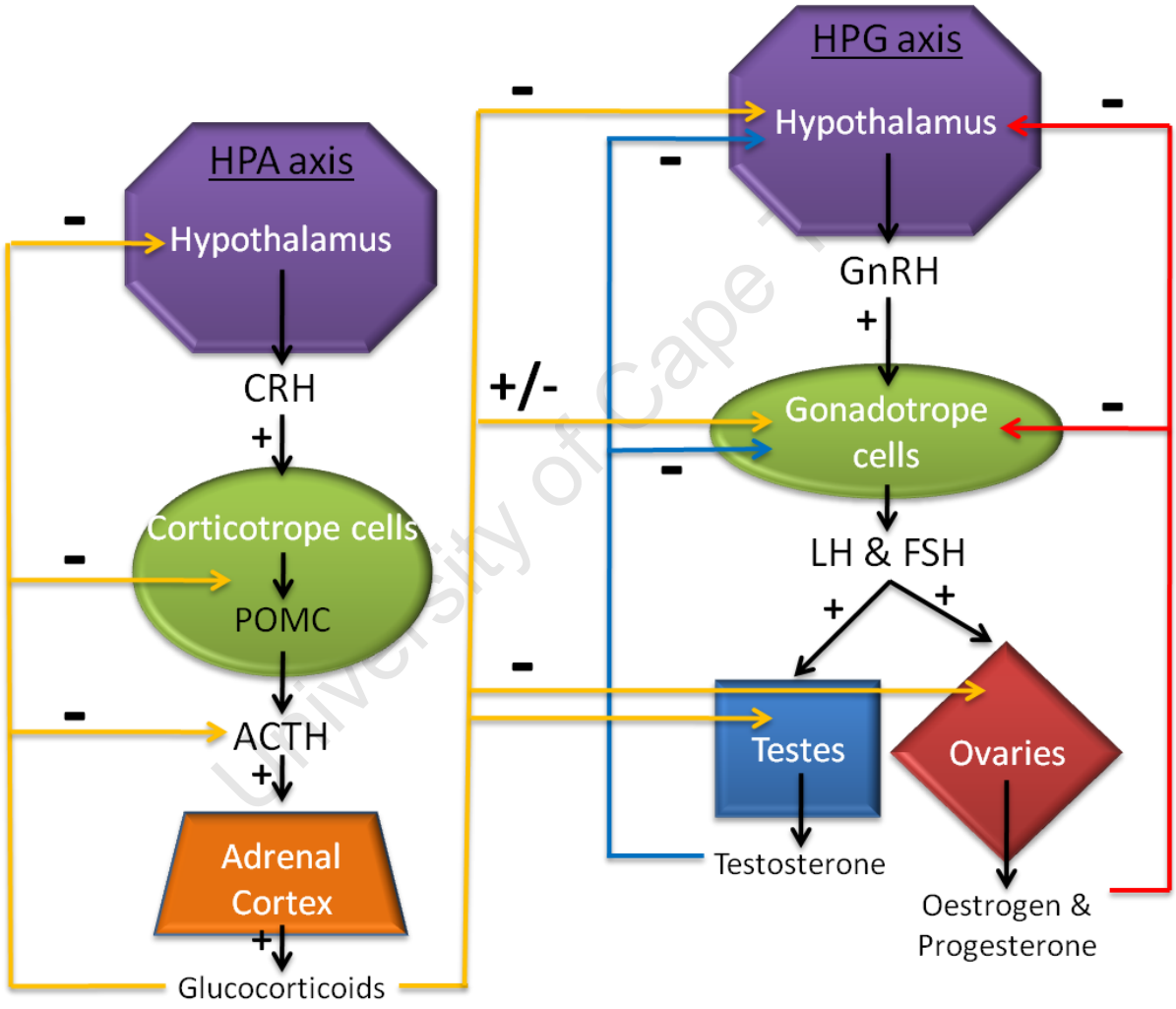

Figure 1.4: HPA/HPG crosstalk. A model representing the major regulatory hormones and endocrine organs involved in HPG-HPA crosstalk signalling. Arrows indicate the points of regulation mediated by hormones along HPG and HPG axes. Arrows indicated positive (+) or negative (-) feedback effects of hormones [figure adapted from Fernandes (2007)].

HPG and HPA crosstalk has also shown to positively influence *GnRHR* expression levels in mouse gonadotrope models, through mechanisms mediated by the GR and GnRHR (Figure 1.4) (Kotitschke *et al.,* 2009). These results presented by Kotitschke *et al,* reveal a molecular insight into how endocrine signalling within the body is functionally integrated to regulate hypothalamic pituitary endocrine signalling. Therefore, factors including metabolism, stress and immune function can influence HPG signalling through receptor crosstalk (Navratil *et al.,* 2009; Kotitschke *et al.,* 2009), supporting the concept that all hypothalamic-pituitary signalling axes are functionally integrated and bi-directionally regulated to maintain homeostasis (Da Silva *et al.,* 1993).

1.2 G-protein coupled receptor family

G-protein coupled receptors (GPCR) form the largest group of membrane receptors involved in membrane signalling events, and encode for the largest gene family in most mammals (Flower, 1999). These cell surface receptors are able to detect a wide array of extracellular stimuli; including peptide and non-peptide neurotransmitters, steroid hormones, growth factors, lipids, ions, odorant molecules and light (Marrinissen and Gutkind, 2001). They mediate signal transduction though G-protein interaction and downstream effector protein activation (Marrinissen and Gutkind, 2001).

1.2.1 GPCR protein structure

GPCRs share a common central domain comprised of 7-transmembrane α-helices, connected by 3 extracellular and 3 intracellular loops. The carboxy (C)-terminal is intracellular and the amino (N)-terminal is extracellular (Bockaert & Pin, 1999). These domains contribute to the specific properties of ligand recognition, receptor activation, G-protein binding and intracellular signal transduction (Kakar *et al.,* 1993).

1.2.2 Rapid G-protein-mediated signalling

GPCR-mediated signal transduction is not dependent on a single biochemical pathway, but rather the integration of an intricate network of rapid intracellular signalling pathways. Signals are mediated through heteromeric G-proteins, consisting of a G_{α}-subunit tightly bound with GDP, and associated with $G_{\beta\gamma}$-subunits. Depending on the class of G-protein coupled to the GPCR, a wide variety of effecter proteins and signalling cascades may be activated in response to a signalling event (Figure 1.5) (Marrinissen and Gutkind, 2001; Nerves *et al.,* 2002).

In response to agonist binding, GPCRs undergo a conformational change that allows the exchange of GDP for GTP on the G_{α} subunit (Figure 1.5). Upon GTP binding, the G_{α} subunit dissociates from the GPCR and the $G_{\beta\gamma}$ dimer (Marinissen and Gutkind, 2001). Here the GTP bound G_{α}, and $G_{\beta\gamma}$ subunits can activate many downstream effecter proteins and subsequent intracellular signalling cascades to regulate diverse biological functions (Ostrom

et al., 2002). Both α and βγ subunits are responsible for the ligand-induced, GPCR-mediated signal transduction.

More than 20 G_{α} subunits are known, and can be divided into four main classes depending on the type of effecter proteins and signalling pathways activated, and include $G_{\alpha s}$; $G_{\alpha i}$; $G_{\alpha q}$ and $G_{\alpha 12/13}$ (Kraus *et al.,* 2001).

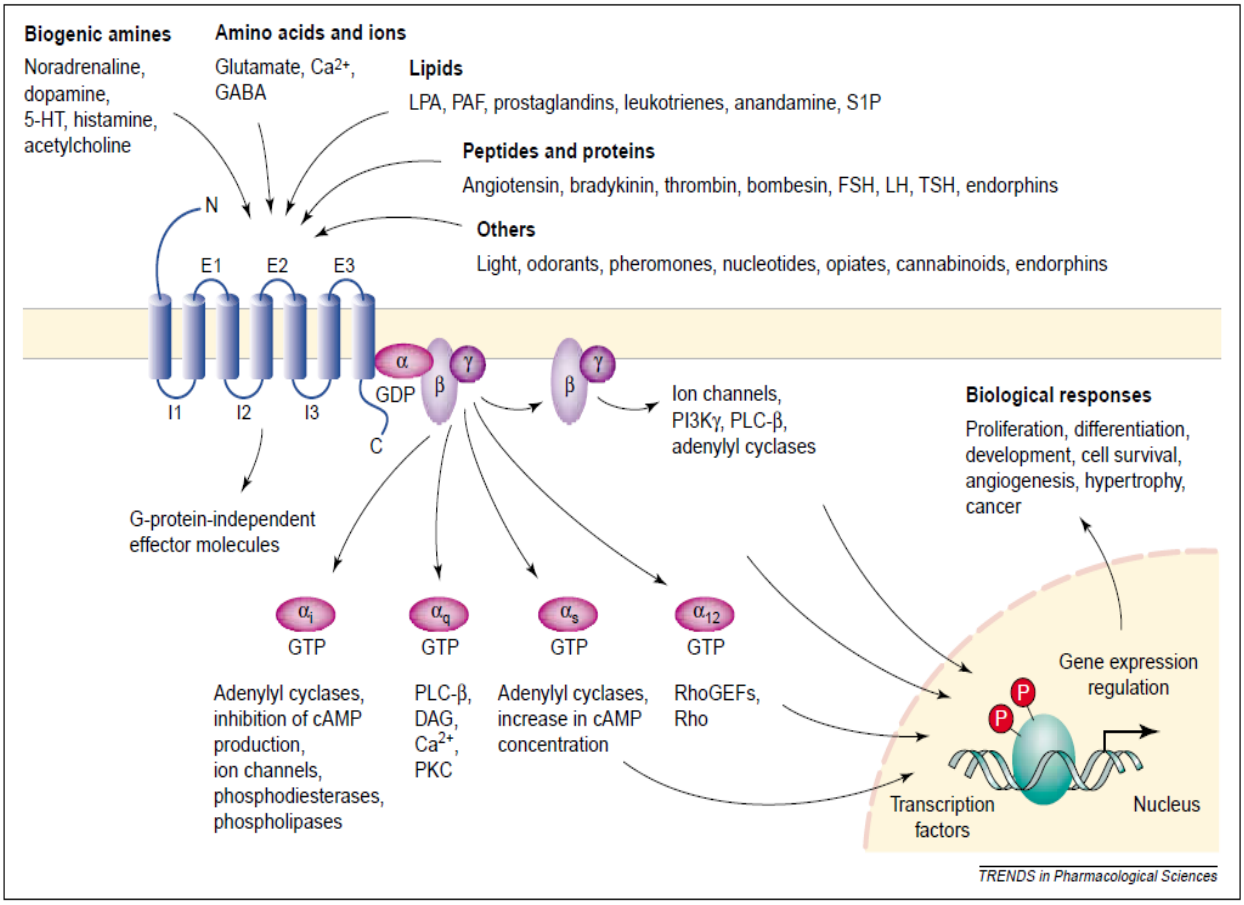

Figure 1.5: The G-protein coupled receptor (GPCR) signalling network. Activation of the GPCR by ligands leads to the dissociation of the GDP-bound G_{α}-subunit and the tightly associated $G_{\beta\gamma}$-subunits which are able to interact with a diverse array of effector molecules involved in signal transduction pathways to regulate key biological responses [from Marinissen and Gutkind (2001)].

The $G_{\alpha s}$ subunit has been shown to activate adenylyl cyclases which increase intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP), resulting in the activation of protein kinase A (PKA) (Beebe *et al.,* 1994; Birnbaumer, 1992). In contrast, the $G_{\alpha i}$ sub-unit has been shown to inhibit adenylyl cyclase activity and activate ion channels

and phospholipases (PLs) (Birnbaumer *et al.,* 1992; Naor *et al.,* 2000). $G_{\alpha q}$ subunits mainly activate phospholipase C (PLC), increasing the intracellular concentration of diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP3) to induce Ca^{2+} mobilization and the activation of protein kinase C (PKC) (Noar *et al.,* 2000; Ostrom *et al.,* 2000). $G_{\beta\gamma}$ dimers mediate signalling pathways that activate phosphoinositide-3 kinase (PI3K), components in the mitrogen-activated protein kinase (MAPK) pathways and PLC (Naor *et al.,* 2000; Hur and Kim, 2002). All the diverse signalling cascades mediated by G-proteins can directly affect gene transcription and result in a number of specific biological responses.

The diverse array of signalling proteins that GPCRs are able to interact with provides insight into how GPCRs are able to be involved in so many diverse and specific signalling pathways. For this reason, GPCR signalling mediates and regulates most biological functions including reproduction, metabolism and cell proliferation, cell differentiation and cell development (Marrinissen and Gutkind, 2001).

1.3 The gonadotropin-releasing hormone receptor (GnRHR)

The GnRHR was first identified in gonadotropes of the anterior pituitary (Hyde *et al.,* 1982). The primary structure of GnRHR was first determined in 1992 through sequencing GnRHR cDNA isolated from an immortalised murine gonadotrope cell line (αT3-1) (Tsutsumi *et al.,* 1992; Reinhart *et al.,* 1992). To date the sequences of mammalian GnRHRs include species such as rat (Eidine *et al.,* 1992), sheep (Brooks *et al.,* 1993), bovine (Kakar *et al.,* 1993), human (Chi *et al.,* 1993) and other non-mammalian species have been described (Millar *et al.,* 2004). All these receptors have been designated as type I GnRHRs, with the mammalian type I GnRHR lacking a C-terminal tail, setting it apart from non-mammalian GnRHRs and other GPCRs (Willar *et al.,* 1999). The primary structure of the human GnRHR is represented in Figure 1.6.

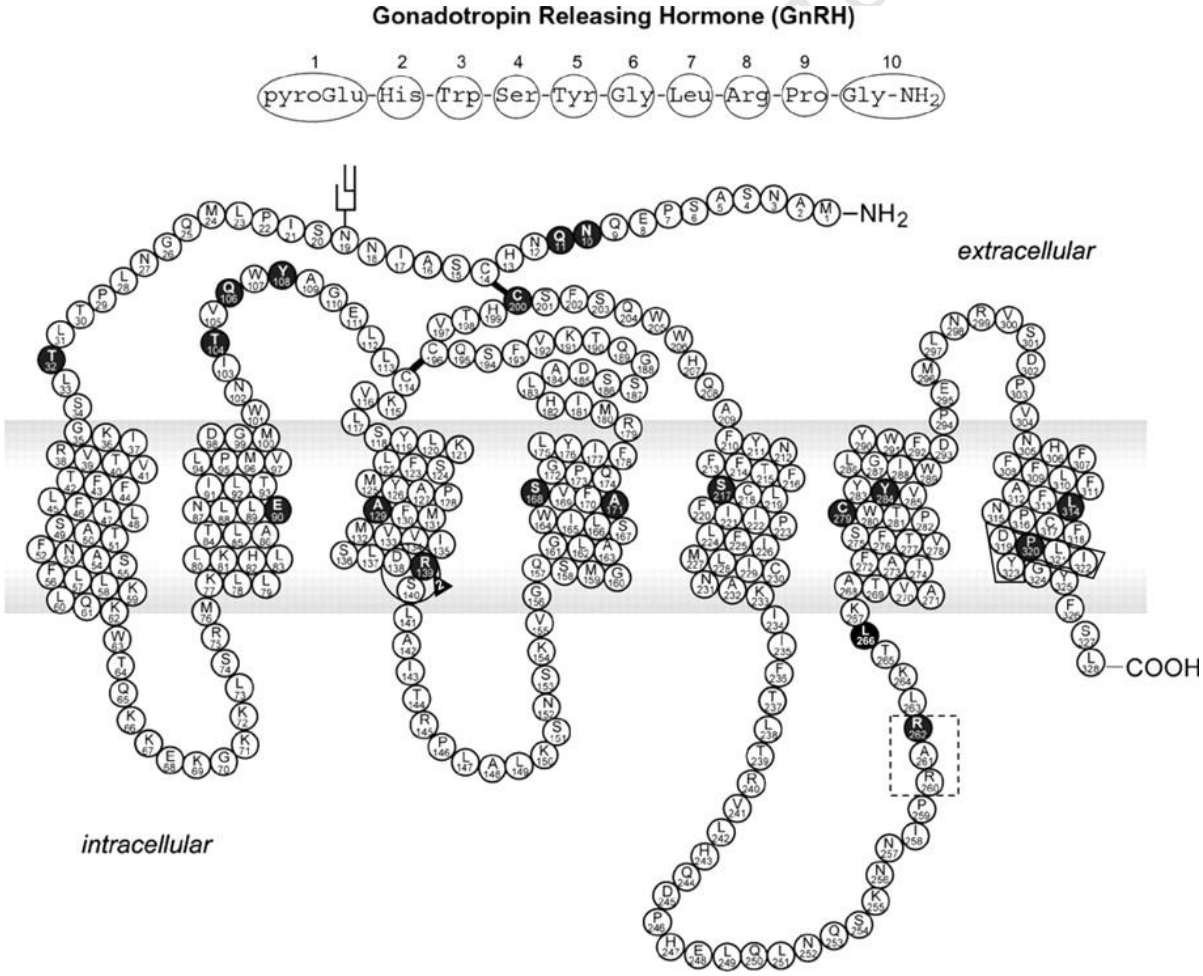

Figure 1.6: The amino acid structure of the GnRHR. Figure shows the ligand and sites of mutations (dark circles) on the human GnRHR that are associated with human disease. Glycosylation sites are represented by branched structures [from Conn *et al.,* (2007)].

Functional type I GnRHR has been detected in reproductive tissues including the pituitary, endometrium, ovary, placenta, uterus and breast. The type I GnRHR has also been detected in other non-reproductive tissues including the prostate gland, liver, heart, skeletal muscle, kidney and peripheral blood mononuclear cells (Kakar and Jennes, 1995; Limonta *et al.,* 2003; Imai and Tamaya, 2000; Imai *et al.,* 1994). Moreover the receptor is also expressed in melanoma and breast tumour cells, highlighting its involvement in cell proliferation (Moretti *et al.,* 2002).

The accumulating evidence in the literature showing the wide spread expression of GnRHR in various extra-pituitary mammalian tissues and cells including reproductive tissues and non-reproductive tissues, suggests a functional role for GnRH and its receptor as an important autocrine and/or paracrine regulator in extra-pituitary tissues (Cheng and Leung, 2005; Hapgood *et al.,* 2005). In male reproductive tissues GnRH acting, via its receptor, has been shown to be involved in spermatogenesis, testis maturation (Cheung and Hearn, 2003) and sperm oocyte interaction (Morales, 1998). In female reproductive tissues GnRH and its conjugate receptor has been shown to regulate the menstrual cycle (Raga *et al.,* 1998), ovarian steroidogenesis (Guerrero, *et al* 1993) and the maintenance of pregnancy (Rama & Rao *et al.,* 2001). Besides reproductive roles, GnRH is also involved in cell proliferation (having anti-proliferative properties) and apoptosis (Cheng and Leung, 2005).

Activation of GPCRs is typically followed by their desensitization and internalization, which involve processes of rapid agonist induced phoshorylation by both second messenger-dependent protein kinases and G protein-coupled receptor kinases (Ferguson. 2001). Studies have shown that the mammalian GnRHR does not undergo rapid homologous desensitization or exhibit agonist-induced phosphorylation (Willars *et al.,* 1999). This supports the concept that mammalian GnRHRs lack serine and threonine residues on the C-terminal tail which are phosphorylated in response to desensitization. Therefore the mammalian GnRHR internalizes slowly via clathrin-coated vesicles and occurs independently of β-arrestin and dynamin (Heding *et al.,* 1998). This unusual resistance to desensitization is essential in mediating GnRHRs anti-proliferative properties with regard to sustained ligand stimulation (Everest *et al.,* 2001).

1.3.1 GnRHR gene structure

The structures for the mouse (Zhou and Sealfon, 1994), rat (Reinhart *et al.,* 1997), human (Fan *et al.,* 1995), pig (Jiang *et al.,* 2001) and sheep (Campion *et al.,* 1996) GnRHR I genes have been characterised. In these species, the GnRHR I gene has a high degree of sequence homology within the coding regions, and consist of 3 exons separated by 2 introns. The intron exon boundaries are conserved across species, but the sizes of the introns, as well as the sequence and length of 5' and 3' untranslated regions (UTRs) differ across species (Figure 1.7). Exon 1 encodes the N-terminal tail and the transmembrane helices (TM) 1, 2, 3 and part of 4. Exon 2 encodes the rest TM 4 and TM 5. Exon 3 encodes TM 6 and 7 (Figures 1.6 and 1.7) (Fan *et al.,* 1994). The GnRHR gene exists as a single copy gene and encodes a 327-328 amino acid protein (Cheng and Leung, 2005).

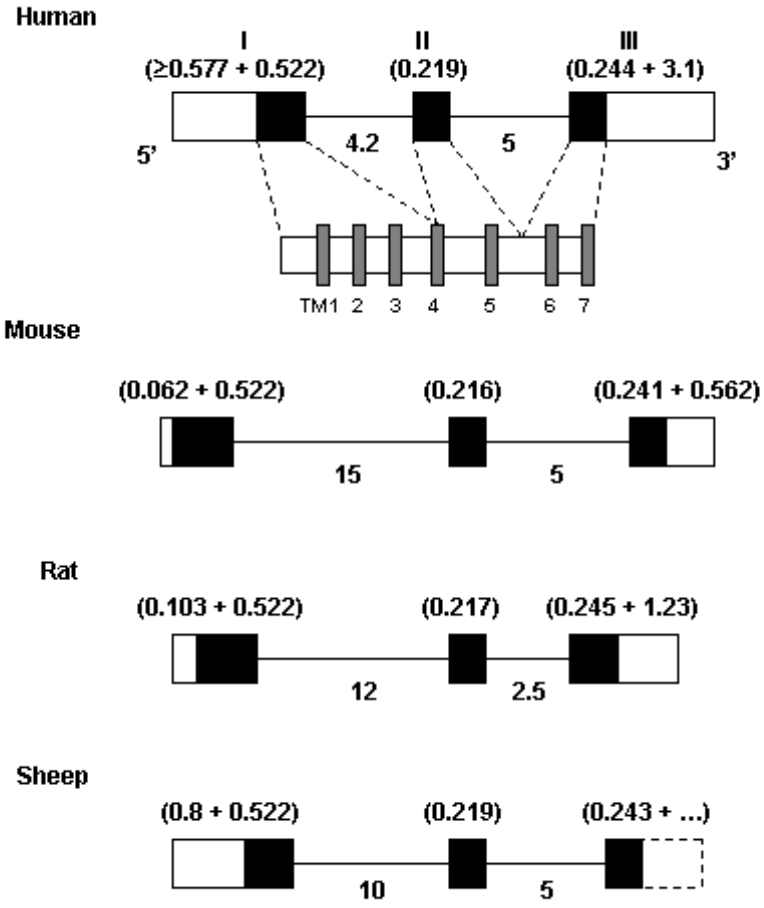

Figure 1.7: Structural organization of the GnRHR I gene in human, mouse, rat and sheep. Exons (I, II & III) are represented by blocks, with portions containing coding sequences shown as dark blocks, and untranslated regions (UTR) shown as light blocks. Sizes of coding, and non-coding portions of exons are indicated above and represented in kilobasepairs. Introns are represented by solid lines, with sizes indicated below, and represented as kilobase pairs [figure adapted from Hapgood *et al.,* (2005)].

1.3.2 GnRHR promoter

The 5' flanking region of the mouse GnRHR gene has been cloned and the major transcriptional start site has been identified 62 nucleotides upstream of the ATG translational start site (Albarracin *et al.,* 1994). While no TATA or CAAT sequences were found in the mouse GnRHR promoter region, several minor transcriptional start sites where identified (Figure 1.8) (Albarracin *et al.,* 1994). Albarracin *et al.* (1994) further showed that a 1.2 Kb mouse GnRHR genomic fragment attached to a luciferase reporter gene appeared to be sufficient in directing high levels of expression in αT3-1 cells. This region appeared to be

pituitary- and gonadotrope-responsive, as low expression occurred in the transfected placental cell line JEG-3 and the kidney fibroblast cell line CV-1 (Albarracin *et al.,* 1994; Kaiser *et al.,* 1997).

Sequencing analysis (Figure 1.8) revealed a consensus activator protein-1 (AP-1) element at the position -336/-330 relative to the transcriptional start site (Albarracin *et al.,* 1994; Kaiser *et al.,* 1997). A gonadotrope-specific element (GSE) like sequence at position -15/-7 has been identified as a binding site for the orphan nuclear steroidogenic factor-1 (SF-1) (Kaiser *et al.,* 1997; Duval *et al.,* 1997a). Other researchers have identified a tripartite enhancer element that regulates cell specific expression comprised of a binding site for SF-1 at -244/-236 bp (Duval *et al.,* 1997b), a consensus AP-1 site at -336/-330 bp and an element referred to as GnRHR activating sequence (GRAS) at -391/-380 bp relative to translational start site (Duval *et al.,* 1997a). GRAS was found to contain overlapping functional elements and binding sites for SMAD, AP-1 and FoxL2 proteins (Ellsworth *et al.,* 2003; Norwitz *et al.,* 2002). In addition a region designated Sequence-underlying Responsiveness to GnRH-1 (SURG-1) has been identified including binding sites for Oct-1 and nuclear factor Y (NF-Y) (Kam *et al.,* 2005). Furthermore a region termed DARE (down-stream activin regulatory element) was found to contain binding sites for LHX2, a member of the LIM homeodomain family (Cherrington *et al.,* 2005), as well as an ATTA element located at -360 bp in the proximal GnRHR promoter where LHX3 was shown to bind *in vitro* and *in vivo* (McGillivray *et al.,* 2005).

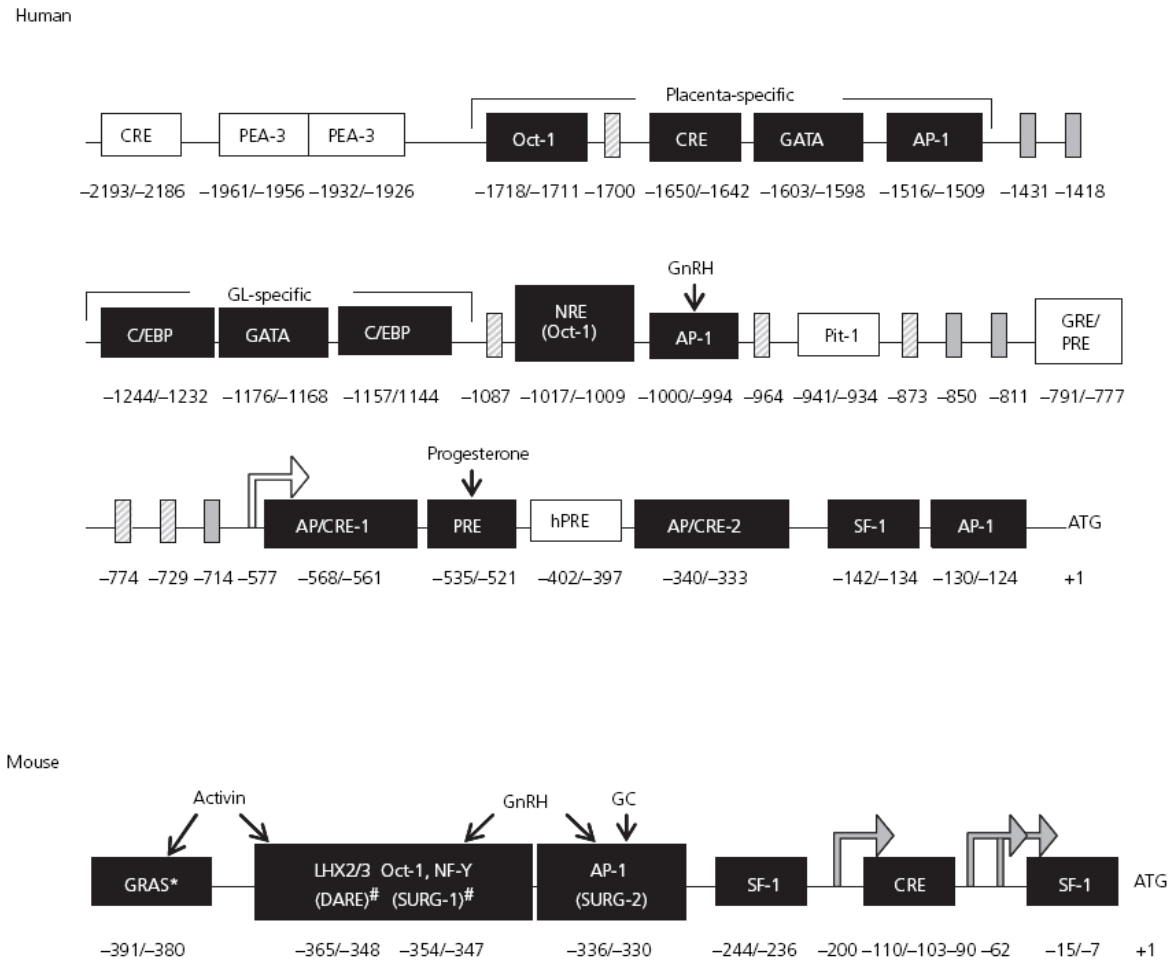

Figure 1.8: Regulatory elements of the GnRH-R I promoter region for the human and mouse gene. Shaded boxes indicate TATA elements, striped boxes indicate CCAAT elements. Functional cis-elements that have been characterised are shown in the black boxes. Putative elements which have been identified by promoter sequence analysis are shown in white boxes. Transcriptional start sites are indicated by arrows and the ATG shows the translational start site. The mouse site GRAS contains binding sites for SMAD, AP-1 and FoxL2 proteins. Figures are not drawn to scale [from Hapgood *et al.,* (2005)].

1.3.3 GnRHR-mediated signalling

Type I GnRHR is responsible for mediating the effects of GnRH signalling in the pituitary and is essential in maintaining gonadotropin serum levels, ensuring fertility (Burns and Matzuk, 2002; Wen *et al.,* 2010). When activated by ligand binding, GnRHR mediates intracellular

signalling cascades that regulate the synthesis and secretion of LH and FSH gronadotropins (Wen *et al.,* 2010).

With regards to the GnRHR ligand, two GnRH isoforms have been found (Neill *et al.,* 2001), GnRH I and GnRH II, which differ by three amino acids (Burns and Matzuk, 2002). Both isoforms have specific affinities for their corresponding receptors. They differ in their tissue expression patterns, potential action, however, have been described in several species including humans (White *et al.,* 1998; Burns and Matzuk, 2002). GnRH II is expressed in several regions of the brain, including the amygdale, caudate nucleus, hippocampus and thalamus, as well as the kidney, bone marrow, prostate, endometrium and ovary (Cheon *et al.,* 2001; Kang *et al.,* 2001). Although *in vitro* experiments indicate GnRH II has anti-proliferative effects on ovarian carcinoma cells (Choi *et al.,* 2001), functional characterisation of GnRH II and its receptor remain unreported in the literature, therefore the role of GnRH II signalling in gonadotropin physiology remains unknown. A second type II GnRHR has been shown to be ubiquitously expressed and down-regulated in multiple tumor cell lines, suggesting its involvement in inhibiting cell proliferation and prompting cell differentiation (Neill *et al.,* 2001)

The nature of G protein-coupled signalling initiated by the GnRHR largely depends on cellular context. For example, it has been demonstrated that the human GnRHR couples to $G_{\alpha q}$ in Chinese hamster ovary-K1 and COS-7 cells (Stanislaus *et al.,* 1998), whereas it couples to $G_{\alpha s}$ in the placenta (Cheng *et al.,* 2000). In mouse pituitary cell lines, the GnRHR activates four MAPK cascades' including the ERK1/2, the c-Jun amino-terminal (JNK), the p38 MAPK and the big MAPK (BMK1/ERK5) pathways to various extends by PKC, Ca^{2+}, and tyrosin kinase-dependent mechanisms (Levi *et al.,* 1998; Roberson and Mulvaney, 1999; Kraus *et al.,* 2001). These signalling cascades mediate the GnRH-targeted gene transcription in rat and mice models (Weck *et al.,* 1998; Saunders *et al.,* 1998; Vasilyev *et al.,* 2002; Call and Wolfe, 1999; Vasilyev *et al.,* 2002).

1.3.4 Regulation of GnRHR mRNA expression

Glucocorticoids have been shown to directly influence GnRHR expression in immortalised gonadotrope cell lines, with the ligand-dependent activation of the GR inducing both GRE reporter-promoter transactivation in αT3-1 cells (McGillivray *et al.,* 2007) and endogenous GnRHR gene expression in LβT2 cells (Kotitschke *et al.,* 2009; von Boetticher, 2008).

The transcriptional effect of GnRH regulating endogenous GnRHR expression has been studied by research groups (Ellsworth *et al.,* 2003). Previous studies from the Hapgood lab have shown that continuous treatment with 100 nM GnRH increases the expression of a synthetic transfected mouse GnRHR reporter-promoter construct by 2.5-fold (Sadie, 2006), while continuous 8 hour treatments of 100 nM GnRH was also shown to up-regulate endogenous LβT2 *GnRHR* mRNA expression (Kotitschke *et al.,* 2009; Sadie, 2006).

In a physiological context, GnRH is released in a pulsatile manner from the hypothalamus to regulate the expression and secretion of the gonadotropin hormones FSH and LH (Levine and Ramirez, 1982). Studies performed by Bedecarrats *et al.,* (2003) have shown that the pulsatile stimulation with GnRH (1 pulse every 30 min) for 10 hours resulted in a 2-fold increase in GnRHR promoter activity and a 2-fold increase in GnRHR numbers on the cell surface (Bedecarrats and Kaiser, 2003). Taken together, these findings suggest that the GnRHR is transcriptionally regulated by both pulsatile and continuous treatment of GnRH in LβT2 cells.

Finally a studied performed by Kotitschke *et al.,* (2009) revealed that Dexamethasone (Dex) and GnRH co-treatments act synergistically in up-regulating endogenous LβT2 *GnRHR* mRNA expression. The study further showed that the effects of Dex and GnRH co-treatment on GnRHR expression are mediated by GR and GnRHR crosstalk, and involves a combination of genomic and non-genomic signalling events to enhance *GnRHR* promoter activity (Kotitschke *et al.,* 2009).

1.4 Nuclear receptor family 3C: steroid hormone receptors

Nuclear steroid receptors play a vital role in gene expression and regulating aspects of physiology, including in reproduction, stress, metabolism and immune function.

Nuclear receptors are ligand-inducible, sequence-specific, DNA-binding proteins that recognise palindromic hormone response elements (HREs) in promoter regions of target genes to regulate gene transcription. They belong to a sub-class of the nuclear receptor super family 3, based on their primary sequence and mechanism for HRE-mediated transactivation (Carson-Jurica *et al.,* 1990). Upon ligand-binding and receptor dimerization, these receptors adopt a head to head orientation when bound to the DNA, allowing for necessary interactions between basal transcription machinery and additional co-activators to drive chromatic transcriptional activity (Lu *et al.,* 2006; Mangelsdorf *et al.,* 1995).

The glucocorticoid, estrogen, progesterone, androgen and mineralocorticoid (GR, ER, PR, AR and MR) receptors all belong to this subclass of modulating receptors, as they share primary and tertiary structural similarity, as well as gene regulatory mechanisms.

1.4.1 Common domains and structure of nuclear receptors

Nuclear receptors are modular proteins all consisting of 3 common domains, an N-terminal domain (NTD), a highly conserved DNA-binding domain (DBD) and a C-terminal or ligand-binding domain (LBD). Within these domains are at least 2 transcriptional activation sub-domains or functions (AF-1 & AF-2) and a hinge region domain (Lu *et al.,* 2006) (Figure 1.9).

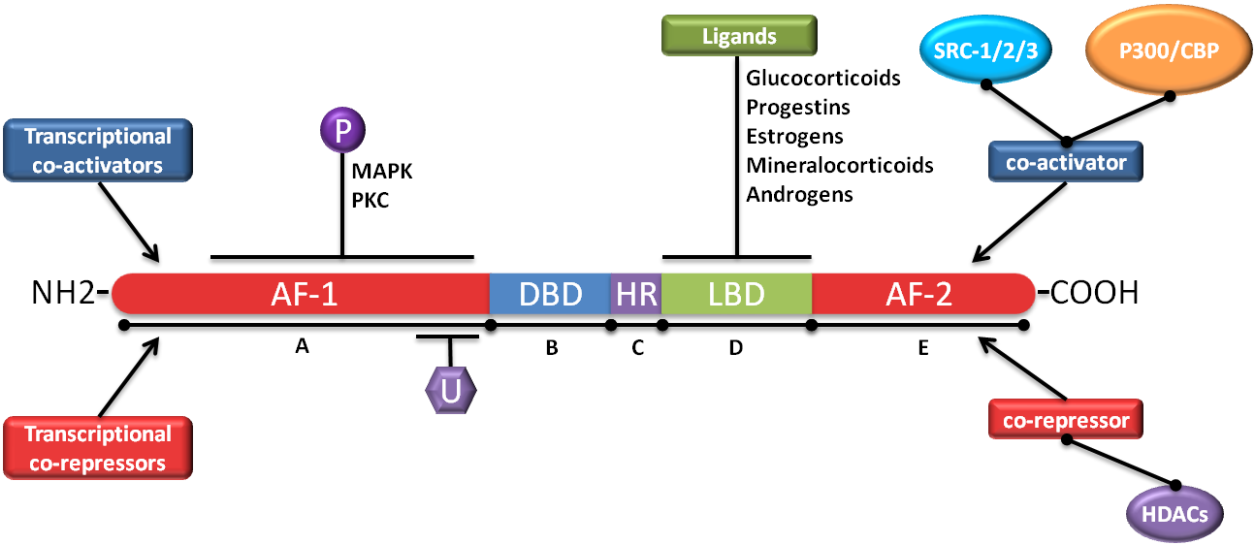

Figure 1.9: The functional domains of nuclear steroid receptors. The domains AF-1 (N-terminal domain), DBD (DNA-binding domain), HR, (hinge region), LBD (ligand binding domain) and AF-2 (C-terminal domain) are represented. Domains where multiple protein-protein interactions occur are shown with arrows. Co-activators include steroid receptor co-activator (SRC) 1/2/3 and the p300/CREB-binding protein (CBP). Co-repressors include histone deacetylases (HDACs). Areas where phosphorylation (P) and ubiquitation (U) occur are shown [figure adapted from Zhou and Cidlowski, (2005)].

AF1 (Figure 1.9, A) resides in the NTD and is important for full transcriptional activity of the SRs (Mangelsdorf *et al.,* 1995; Kumar *et al.,* 2003*)*. This domain interacts directly with basal transcription machinery and multiple cofactors involved in regulating transcriptional activity (Dahlman-Wright *et al.,* 1995; McEwan *et al.,* 1993), and contain multiple Ser/Thr phosphorylation sites which are modified by various protein kinases (Weigel, 1996; Lange *et al.,* 2000; Rochette-Egly *et al.,* 2003).

The central DBD (Figure 1.9, B) consists of 66-68 highly conserved amino acids and 9 perfectly conserved cysteine residues. This primary sequence forms two zinc finger α-helix motifs, where zinc is coordinated into tetrahedral geometry by four cystines, resulting in correct orientation of two conserved α-helixes. Upon head-to-head homodimerization, one α-helix of each SR is oriented into two adjacent major grooves for specific palindromic DNA

sequence recognition of HREs (Lu *et al.,* 2006). The other α-helix is important for protein-protein interaction for steroid receptor dimerization (Dahlman-Wright *et al.,* 1991; Lu *et al.,* 2006).

On the N-terminal side of the LBD there is a flexible hinge region (Figure 1.9, C) that is responsible for ligand-binding and ligand-induced changes in conformational states. Upon ligand-activation, a nuclear localisation sequence is exposed in this region that promotes nuclear transportation (Picard & Yamamoto 1987).

The LBD (Figure 1.9, D) consists of 11 α-helixes and 4 β-sheets which fold into a three-dimensional ligand binding pocket (Zu *et al.,* 2006). There are three structural features that ensure ligand selectivity. First is a unique hydrogen bond network between receptor and bound ligand that establishes specific ligand recognition. Second is the shape of the ligand and the topology inside the binding pocket which enhances selectivity. Third is the relative position of the binding pocket within the receptors' LBD. Variations in these three factors contribute to ligand-specific binding characteristics of each SR (Zu *et al.,* 2006).

The AF-2 sub-domain is found on the C-terminal end of the LBD (Figure 1.9, E). This co-activator binding cleft on the surface of the LBD has been shown to recruit a wide variety of co-activators that determine transcriptional activity. Specific charged residues and intermolecular interactions facilitate relative cofactor recruitment of each SR. (Williams and Sigler, 1998; Matais *et al.,* 2000; Bledsoe *et al.,* 2002; Li *et al.,* 2005). A common leucine-rich LxxLL motif is found among AF-2 sub-domains for most of the SRs, which is believed to mediate the interactions between SRs and co-activators (Lonard and O'Malley, 2005), whereas the AR contains an FxxLF motif. This motif has been found to interact with multiple co-activators including steroid receptor coactivator-1 (SRC-1) (or NcoA-1), SRC-2 (TIF-2 or GRIP-1, NcoA-2), SRC-3 (RAC3, ACTR, AIB1,P/CIP and TRAM) (Heery *et al.,* 1997; Le Douarin *et al.,* 1996; Savkur and Burris, 2004; Torchia *et al.,* 1997), and the p300/CBP "docking platform" (Nicolaides *et al.,* 2010).

1.4.2 Classical nuclear signalling by steroid receptors

The genomic actions of SRs in mediating gene transcription have been examined extensively. Upon binding steroid, SRs in target cells become activated through a process involving conformational changes, dissociation from protein chaperones, nuclear transport and binding to palindromic steroid hormone response element (HRE) sequences in gene target promoters (Figure 1.10) (Tsai *et al.,* 1994). Once directly or indirectly bound to the DNA, SRs can mediate their transcriptional regulation through transactivation or transrepression mechanisms.

The majority of unbound SRs reside in the cytoplasm, where they are found in a complex with chaperone proteins including heat shock proteins (HSP90, HSP70), immunophilins, and p23 (Pratt *et al.,* 2003). These interactions are required for proper protein folding and the assembly of a stable SR-HSP heterocomplexes, promoting ligand binding and maintaining cytosolic location (Zu *et al.,* 2006).

Upon ligand-binding, the receptor undergoes a conformational change that causes dissociation from chaperones and translocation to the nucleus (Weigel and Moore, 2007). Here it binds directly to promoter regions of target genes though HRE-protein interactions. HREs consist of inverted DNA sequence repeats of TGTTCT for the GR PR and AR, and TGACC for the ER (Parker, 1990). SRs can also indirectly interact with the DNA through protein (SR)-protein (transcription factor) interactions.

SRs can modulate gene transcription independent of HRE binding through protein-protein (transcription factor-SR) interactions, referred to as tethering. These interactions can disrupt or activate expression of target genes by blocking or enhancing the assembly of transcription machinery to the promoter respectively. Examples of transrepression and transactivation models are seen with the protein-protein interaction of the GR with nuclear factor–kappa B (NFκB) present in cytokine promoters, and the binding to Stat 5 on the βcasein gene, respectively (Kassel and Herrlich, 2007).

DNA-bound SRs alter the transcriptional activity of target gene promoters, through the recruitment of co-activator or co-repressor proteins. These co-factors are devoid of DNA-binding characteristics, and are recruited through protein-protein interactions via the AF-1 and AF-2 sub-domains of SRs. Several co-activators form a bridge between DNA-bound SRs and the transcriptional initiation complex, and facilitate RNA polymerase II activity (Meckenna *et al.,* 1999; Meckenna *et al.,* 1999; Meckenna *et al.,* 2002; Auboeuf *et al.,* 2002).

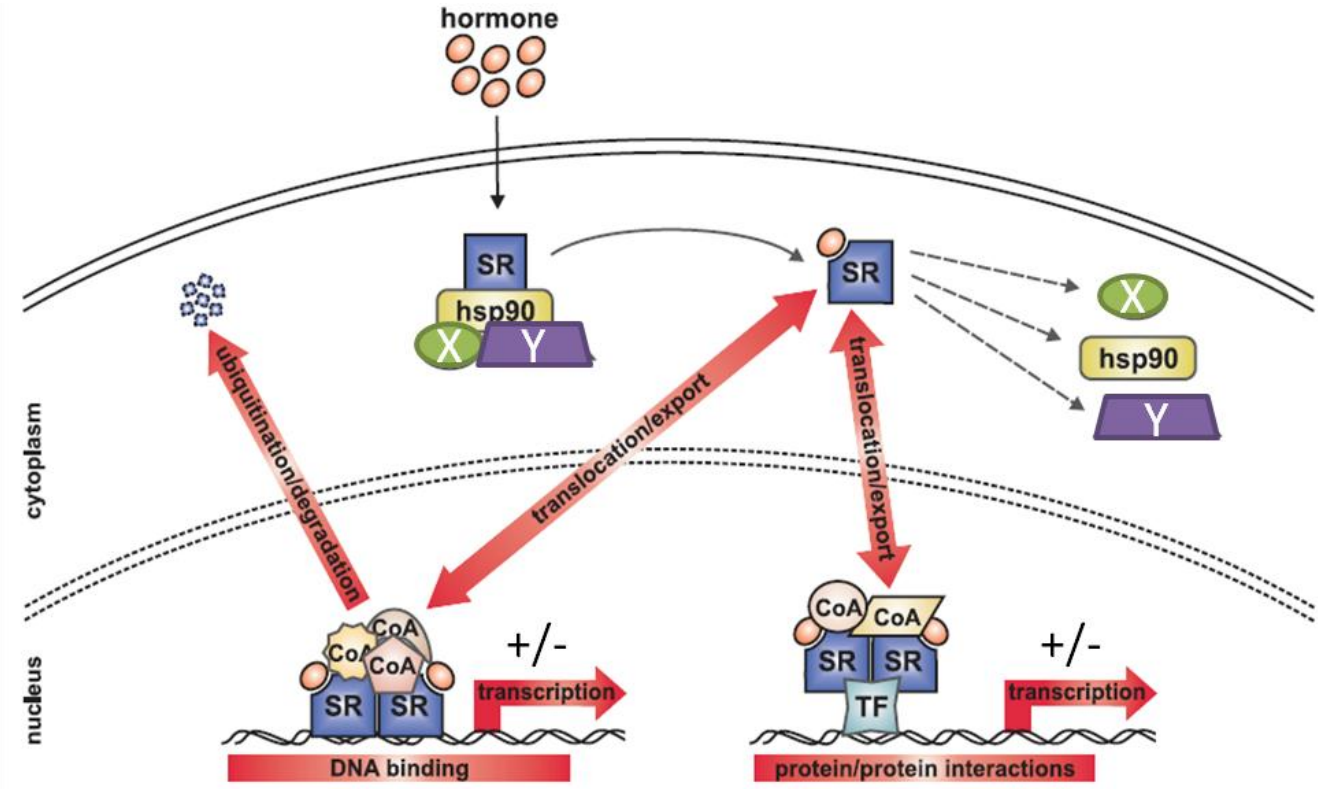

Figure: 1.10. Mechanisms for classical SR action. SR monomers complex with chaperone proteins such as heat shock proteins (hsp) 90, p23 and immunophilins (X & Y). After hormone binding, the receptor undergoes a conformational change, dissociates from chaperones, and translocates to the nucleus where it binds to DNA through HRE interactions and/or interacts with other proteins including co-activators (CoA) or transcription factors (TF) to regulate transcription. Aspects of SR function shown to be regulated by posttranslational modifications (i.e. phosphorylation) are highlighted in red. Sub-cellular localisation of SRs is a dynamic process, with both nuclear translocation and export modulated by phosphorylation. Phosphorylation can also affect DNA-binding affinity of SRs, interactions with other transcription factors and transcriptional activity. SR stability is influenced by ubiquitination and degradation [adapted from Weigel and Moore, (2007)].

Three groups of co-activators have been found to interact with SRs. The first are the p300 and homologous cAMP-response element binding protein (CBP), which form the central position and serve as macromolecular "docking platforms" for SRs, CREB, AP-1, NFκB, p53, Ras-dependent growth factor, and signals inducers and activators of transcription (STATs). Second is the pS300/CBP-associated factor (p/CAF), which is a broad transcription factor that interacts with p300/CBP complex. Third are the p160 family of co-activators; SRC-1, SRC-2 and SRC-3, which interact with the AF-2 domain of SRs through signature LxxLL motifs located on the co-activators. These co-activators have intrinsic histone acetyltransferase activity (HAT) promoting chromatin decondensation, thereby allowing access for the transcription initiation complex (Nicolaides *et al.,* 2010; Mckenna *et al.,* 2002; Auboeuf *et al.,* 2002; Heery *et al.,* 1997: Beato *et al.,* 2000) All these factors are responsible for stabilizing the transcription initiation complex, and promote RNA polymerase II activity.

Post translational modifications including phosphorylation, acetylation, and ubiquitination can affect SR stability, turnover, sub-cellular localisation, ligand affinity, DNA-binding, protein-protein interactions, specificity and extent of target gene-promoter activity (Nicolaides *et al.,* 2010; Weigel and Moore, 2007a; Weigel and Moore, 2007b). The AR, GR, ER and PR have all been shown to be phosphorylated on multiple sites along the AF-1 sub-domains through the activity of specific kinases. Therefore SR activity can be modulated in a phosphorylation-dependent manner brought about through non-genomic signalling cascades (Red arrows in Figure 1.10) (Weigel and Moore, 2007).

Steroid hormones have also been reported to stimulate non-genomic signalling events including second messenger production, ion channels, and protein kinase cascades. These actions happen on a time scale of seconds, and are not affected by inhibitors of gene transcription (Limbourg and Lia, 2003; Losel *et al.,* 2003). These rapid effects of steroid hormones have been mimicked with cell impermeable steroid-protein conjugates, suggesting the event is initiated at the cell membrane and is distinguishable from the classical nuclear actions of steroids (Cato *et al.,* 2002; Norman *et al.,* 2004; Valverde and Parker, 2002; Cheskis, 2002; Watson and Gametchu, 1999; Losel and Wehling, 2003; Revelli *et al.,* 1998; Nemere *et al.,* 2003). Therefore these rapid actions of steroids, independent of

gene transcription, have been termed non-genomic, as opposed to steroids' conventional genomic or direct effect on gene transcription in the nucleus.

1.5 The estrogen receptor (ER)

The ER ligand 17β-estradiol (E2) is an important neurotrophic factor in brain development and differentiation, and has a primary role in transmitting feedback to GnRH neurons in the hypothalamus and to lactotropes in the pituitary (Edwards, 2006). The ovary is the major site of synthesis and secretion for E2, and is tightly regulated by the HPG signalling axis (Figure 1.2). E2 regulates the synthesis and secretion of a number of pituitary hormones that play vital roles in HPG signalling and HPG signalling feedback. E2 regulates GnRH expression and secretion in the hypothalamus, and is involved in the transcriptional regulation of gonadotropin hormones LH and FSH in gonadotrope cells through negative feedback mechanisms (Ng *et al.,* 2009). E2 also has simulative effects on lactotrope proliferation (Lieberman *et al.,* 1978; Lieberman *et al.,* 1981), mediated by hormone-bound ERα up-regulating multiple growth factors, growth factor receptors and proteins involved in cell cycle progression (Katzenellenbogen *et al.,* 2009).

The effects of E2 are mediated by their intracellular receptors. The major ER isoform is a 66 kDa protein termed ERα, which is essential in regulating both reproductive (Pfaff *et al.,* 1994), and non-reproductive processes including: skeletal physiology (Frank. 1995), tumour development and growth (Auchus *et al.,* 1994), and cardiovascular function (Farhat *et al.,* 1996). This intracellular receptor has been found in a number of mammalian tissues including male and female reproductive tracks, female mammary glands, bone, the cardiovascular system and regions of the brain including the hippocampus, hypothalamus, preoptic area, amygdale, cerebellum, cerebral cortex and pituitary gonadotropes (including LβT2 and GN11 cell lines) (Couse *et al.,* 1997; Ng *et al.,* 2009; Edwards, 2006).

1.5.1 The ERα gene and protein structure

The cDNA for ERα was first cloned in 1986 (Green *et al.,* 1986) and the genomic sequence was later described (Ponglikitmongkol *et al.,* 1988). The ERα gene consists of 8 exons spanning 140 kb of chromosome 6 (Gosden *et al.,* 1986). The ERα cDNA shows a high level of sequence homology between human and chicken (Krust *et al.,* 1986), rat (Koike *et al.,* 1987) and mouse (White *et al.,* 1987) ERα transcripts.

Evidence has shown that there are multiple promoters present upstream to the ERα gene (Treilleux *et al.,* 1997; Hodin *et al.,* 1989; Kastner *et al.,* 1990), highlighting the potential for tissue-specific regulation and the control of ER variant mRNA expression. Several attempts to characterise the ERα promoter has been performed mainly in breast cancer and HeLa cell lines (Treilleux *et al.,* 1997; Schuur *et al.,* 2001; deConinck *et al.,* 1995; Cohn *et al.,* 1999; McPherson *et al.,* 1999; Tanimoto *et al.,* 1999; Penolazzi *et al.,* 2000). These studies show the presence of an AP-1 site (Tang *et al.,* 1997), a half-PRE site (Amicis *et al.,* 2009), and a palindomic binding site for the estrogen receptor transcription factor (ERF-1) (McPherson *et al.,* 1997). ERF-1 has been shown to be a member of the AP-2 family of developmentally regulated transcription factors and is likely involved in regulating the expression of genes characteristic of the breast cancer phenotype (McPherson *et al.,* 1997; McPherson *et al.,* 1999).

The ERα promoter has been shown to be down-regulated in response to E2 in MCF-7 cells, while up-regulated in response to E2 in T47D, ZR-75 and EFM-19 cells (Donaghue *et al.,* 1999). This suggests that the transcription factors present within a cell; rather than the selective use of a specific promoter, determines whether ERα mRNA expression is increased or decreased in response to E2 (Donaghue *et al.,* 1999; Castles *et al.,* 1997; Pakdel *et al.,* 1989; Treilleux *et al.,* 1997). ERα promoters have no TATA-box, CCAAT-box or GC-box sequences. Multiple transcription start sites have been identified (Kos *et al.,* 2001), with three half EREs being located and shown to be responsible for the E2 inducibility of the ERα promoter (Treilleux *et al.,* 1997).

Many studies have shown that the ERα promoter region is a large and complex regulatory promoter, comprised of many cis-elements. However, many questions still remain unanswered with regard to the function of multiple promoters, suggesting that further research is required in order to assess how the ERα promoter is regulated at the level of pituitary gonadotropes.

Two major ER isoforms that mediate the genomic effects of E2 in mammalian tissues, referred to as the ERα and ERβ, are encoded by two different genes (Kuiper *et al.,* 1996). The LBD and DBDs of the two ER isoforms are well conserved both at the amino acid level and structurally. The major difference lies in a lack of N-terminal homology (Figure 1.11).

Both isoforms recognise similar HREs and respond similarly to E2. However, there are differences in DNA-binding affinity and specificity for pharmacological ligands (Cowley and Parker, 1999). Although ERα and ERβ are co-expressed in target tissues, they also exhibit differential tissue expression patterns and are functionally distinct, with ERα being the more potent transcriptional activator. Cells lines that express both ERs appear to show ERβ having the role of an attenuator for ERα activity (Hall and McDonnell. 1999).

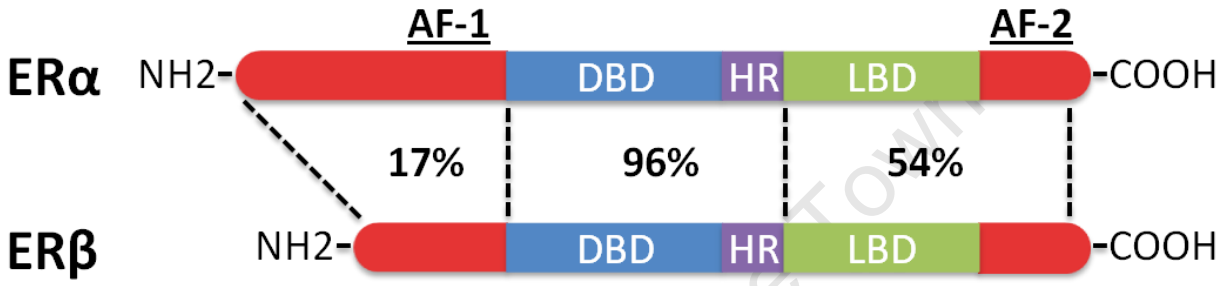

Figure 1.11: Primary structure comparison between the two estrogen receptor isoforms α and β. LBD (Ligand-binding domain); DBD (DNA-binding domain); HR (hinge region); and AF (transcriptional activation domains 1 and 2) are shown. The percentages indicate the amino acid sequence homology between specific domains of the ERα and ERβ. Illustration not to scale [adapted from Edwards (2006)].

An additional isoform of the ER termed truncated estrogen receptor product-1 (TERP-1) has no independent activity, yet it can stimulate the activity of conventional ERα at low ratios and inhibit the activity of ERα and ERβ in the pituitary at high ratios of 1:1 or greater (Resnick *et al.,* 2000). The expression of TERP-1 is limited to the pituitary (Friend *et al.,* 1995; Demay *et al.,* 1996). All three ER isoforms have been found in rat, mice and humans species (Kuiper *et al.,* 1996; Tremblay *et al.,* 1996; Mosselman *et al.,* 1996).

1.5.2 Regulation of ER mRNA expression by hormones

The anti-estrogenic action of progesterone (P4) has previously been established, with liganded PR recruiting specific repressor complexes to the *ERα* promoter region and down-regulating *ERα* expression in MCF-7 breast cancer cells (Amicis *et al.,* 2009). This P4-mediated inhibition of *ERα* mRNA expression in reproductive tissues has been shown by a number of research groups in breast cancer cells (Read *et al.,* 1989; Alexander *et al.,* 1990), and in the uterus (Mester *et al.,* 1974; Hsueh *et al.,* 1990). Futhermore PR-B has been shown to recruit a repressor complex containing nuclear receptor co-repressor (NCoR) to a half-PRE site on the ERα gene promoter region in MCF-7 human breast cancer cells (Amicis *et al.,* 2009).

Studies have shown that E2 regulates ERα mRNA expression in reproductive tissues (Donaghue *et al.,* 1999; Castles *et al.,* 1997; Treilleux *et al.,* 1997). In most cases E2 has been shown to up-regulate ERα mRNA expression in reproductive cell lines including T47D, ZR-75 and EFM-19 cells (Donaghue *et al.,* 1999). It is this auto-regulation of ER expression which may contribute to its over-expression in some breast cancer tumors (Castles *et al.,* 1997). On the other hand, E2 has been shown to down-regulate ERα mRNA expression in MCF-7 cells (Donaghue *et al.,* 1999) and down-regulate ERα and ERβ mRNA expression in primary rat pituitary cells (Schreihofer *et al.,* 2000). Schreihofer *et al.,* (2000) also showed that *TERP-1* mRNA expression was increased in response to E2, further suppressing ER-mediated acftivity in the rat pituitary (Schreihofer *et al.,* 2000).

1.5.3 ER-mediated signalling

The ER is known to mediate gonadotrope signalling. The effects of estrogen have been shown to directly repress GnRH expression in GT1-7 neurons via mechanisms dependent on ER protein (Roy *et al.,* 1999). The ERα has also been shown to be activated ligand-independently in response to GnRH treatment in the LβT2 cell line (Chen *et al.,* 2009). Chen *et al.,* (2009) further showed the GnRHR-mediated phophorylation of the ERα coincides with an increase in binding to the co-activator, p300/CBP-associated factor (PCAF) and transcriptional activation of fosB mRNA expression (Chen *et al.,* 2009).

Interestingly E2 has been shown to affect GR-mediated signalling in MCF-7 and T47D breast cancer cells (Zhang *et al.,* 2009). Zhang *et al.,* showed that E2 does not effect GC-induced GR nuclear translocation, but instead reduced ligand-induced GR phosphorylation at Ser-211, associated with the active form of GR. The research group further showed an increase in protein phosphatise 5 (PP5) expression (mediating the dephosphorylation of GR at Ser-211), in response to E2 (Zhang *et al.,* 2009). This study suggests a crosstalk between estrogen and glucocorticid signalling, with estrogens indirectly regulating GR activity.

1.5.4 Rapid non-genomic actions of E2

The rapid non-genomic effects of E2 were first identified in 1967 (Szego and Davis, 1967), and these rapid effects were mimicked by cell impermeable steroid-protein conjugates, suggesting a plasma membrane initiated event distinguishable from intracellular or nuclear actions of E2 (Cato *et al.,* 2002). For this reason, a candidate orphan GPCR was thought to mediate the rapid effects of E2. The orphan G-protein coupled receptor 30 (GPR30) has been shown to localise either to the plasma membrane (Filardo *et al.,* 2000) or to the endoplasmic reticulum (Revankar *et al.,* 2005).

A recent study showed that E2 failed to bind to GPR30, and a GPR30 agonist (G1) did not stimulate oestrogen actions in mammary gland or reproductive organs (Otto *et al.,* 2008). Therefore, these results suggest that GPR30 is not an ER.

On the other hand, there is increasing evidence that the nuclear ER translocates to the cell membrane to mediate the non-genomic effects of E2. Studies of isolated cells from combined ERα and ERβ knockout transgenic mice show a lack of all E2 binding at the plasma membrane, and failed to respond to E2 by rapidly activating signal transduction pathways (Pendram *et al.,*2006). The ER can interact with a wide variety of signalling proteins capable of initiating kinase cascades, thereby influencing biological responses. The ERα was found to interact with caveolin-1 through Serine 522 (Razandi *et al.,* 2003), further supporting the role of ER in non-genomic signalling. Caveolin-1 is a required structural protein for ERα to interact with caveolae rafts at the plasma membrane (Razandi *et al.,* 2002). It is also believed that caveolin-1 mediated membrane localisation allows ERα and ERβ to associate with, and activate G_{α} and $G_{\beta\gamma}$ proteins to activate Ca^{2+} fluxes and cAMP generation, and

activate proximal kinases (Src, PI3K) and distal kinases (ERK) (Kumar *et al.,* 2007). It is these signals that are proposed to result in the rapid non-genomic effects of E2, including the post-translational phosphorylation of many other signalling proteins, modulating cell transcription and proliferation.

1.6 The progesterone receptor (PR)

Broadly speaking, the major roles of P4 in mammals are 1) in the uterus and ovary: regulating the release of mature ooctyes, facilitation of implantation, and the maintenance of pregnancy; 2) in the mammary gland: lobular and alveolar development in preparation of milk secretion, and suppression of milk protein synthesis; and 3) in the brain: mediation of signals that are require for sexual behaviour and the indirect regulation of key HPG target genes (Graham and Clarke, 1997).

The actions of P4 are mediated through the binding to its conjugate receptors, the full length PR-B and N-terminally truncated PR-A isoforms (Figure 1.12) (Kraus *et al.,* 1993). The PR isoforms are members of the type I sub-family of the nuclear hormone receptors 3C family, and are classified as ligand-activated transcription factors (Carson-Jurica *et al.,* 1990).

1.6.1 The PR gene and protein structure

The single-copy human PR gene uses separate promoters and transcriptional start sites to produce the two isoforms PR-B and PR-A (Kastner *et al.,* 1990), which are identical except for an additional 165 amino acids present only in the N-terminus of PR-B (Figure 1.12). Although the separate promoters of PR-A and PR-B are E2 inducible, they do not contain any consensus palindromic estrogen response elements (EREs). While a “half-palindromic” ERE might be involved in the E2 responsiveness in the promoter for A, no candidate ERE could be found in the promoter for B (Kastner *et al.,* 1990).

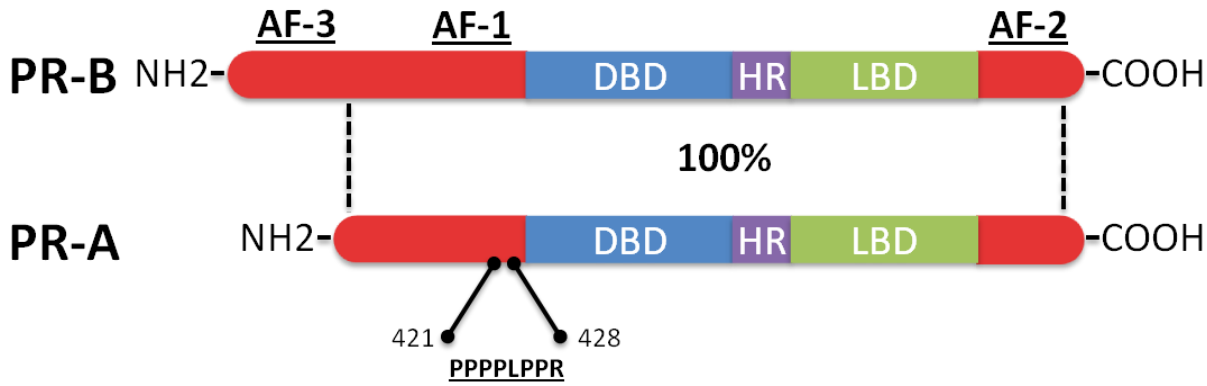

Figure 1.12: Primary structure comparison between the PR isoforms B and A. LBD (Ligand binding domain); DBD (DNA binding domain); HR (hinge region); and AF (transcriptional activation domains 1 and 2) are shown. The percentage indicates the amino acid sequence homology between the PR-B and PR-A. Illustration note to scale. Adapted from Edwards, 2006.

PR-B and PR-A are 116 kDa and 97 kDa proteins, respectively, which interact with specific PREs in the promoter regions of target genes including *c-myc* (Moore *et al.,* 1997), *fatty acid synthetase* (Chalbos *et al.,* 1987), *FSH* (An *et al.,* 2009) and the *mouse mammary tumour virus* (MMTV) promoter (Haraguchi *et al.,* 1997). While PR-B tends to be a stronger activator of target genes, PR-A can act as a dominant repressor of PR-B activity (Vegeto *et al.,* 1993), suggesting that high PR-A expression may reduce P4 responsiveness of PR-B. The repressive role of PR-A has also been shown to diminish the responsiveness of the GR, ER, MR and AR to their appropriate ligands (McDonnell *et al.,* 1994; McDonnell and Goldman. 1994)

Each PR isoform contains a C-terminal ligand binding domain, a DNA-binding domain, a hinge region, and at least two AF sub-domains, located in the lignad-binding domain (AF-1) and in the N-terminal domain (AF-2). The N-terminal of the PR-B contains an additional AF-3 sub-domain within the unique 164-amino acid upstream segment, which contributes to the stronger transcription activity of PR-B (Giangrande *et al.,* 1999; Li and O'Malley, 2003).

PR-B contains a total of 14 known phosphorylation sites (Zhang *et al.,* 1994; Zhang *et al.,* 1995; Zhang *et al.,* 1997). Serines at positions 81, 162, 190 and 400 are defined as "basal" sites that are constitutively phosphorylated in the absence of ligand. Serines 102, 294 and

345 are ligand-inducible phosphorylation sites that are maximally phosphorylated 1-2 hours after P4 treatment (Zhang *et al.,* 1995). Although the role of PR phosphorylation is not fully understood, it may influence co-factor interactions and PR turn-over (Lange *et al.,* 2000). Additionally MAPK-dependent Ser294 phosphorylation has been shown to be required for rapid nuclear translocation of unliganded PR, suggesting MAPK signalling may regulate PR activity by altering nucleo-cytoplasmic shuttling (Qui *et al.,* 2003).

Both PR isoforms are expressed in the female reproductive tract, mammary gland, brain, pituitary gland and specifically in gonadotrope cells (Mangal *et al.,* 1997; Soyal *et al.,* 2005). The PR isoforms are major regulators of the HPG signalling axis by differentially regulating the transcription of the two gonadotropins (LH and FSH) (Figure 1.2) during the menstrual cycle (Thackray *et al.,* 2006; Lu *et al.,* 2006). PR-A and PR-B are co-expressed in most target tissues. However, their ratio can vary considerably depending on cell type or physiological conditions, suggesting differential PR expression contributes to cell-specific response (Shyamala *et al.,* 1998). Transgenic mice models have shown that PR-A has a predominant physiological role in mediating the actions of P4 in the uterus and ovary, whereas PR-B is more important in the mammary gland and pituitary (Mulac-Jericevic *et al.,* 2000; Mulac-Jericevic *et al.,* 2003)

1.6.2 PR-mediated signalling

Studies have highlighted the importance of P4 signalling in the regulation of LβT2 responses. For instance, the Mellon Lab has shown with over-expressed PR protein, the differential effects of P4 treatments on both LHβ and FSHβ expression in the LβT2 cell line (Thackray *et al.,* 2006a; Thackray *et al.,* 2006b). P4 was shown to inhibit basal and GnRH-induced induction of *LH*β, while FSHβ was shown to be up-regulated in response to P4 treatments. Both these responses occur in a homone- and receptor-dependent manner, yet the mechanisms of liganded PR differ. In the case of LHβ repression, the PR was shown not to bind to the DNA directly, but rather recruited to the LHβ promoter region through protein-protein interactions with transcription factors (Thackray *et al.,* 2006a). FSHβ expression was induced via direct binding of the liganded PR to the FSHβ promoter region (Thackray *et al.,* 2006b). These studies highlight P4 signalling feedback at the level of pituitary gonadotropes

ensures differential regulation of the gonadotropin genes. P4 signalling has also been shown to modulate GnRH signalling at the level of the hypothalamus, as seen with P4's negative effects of GnRH release (Slieter *et al.,* 2009; Skinner *et al.,* 1998).

In the brain, PR expression has been linked with lordosis behaviour (behaviour associated with sexual reproduction). Transgenic mice with no functional PR show a complete lack of lordosos response after hormone administration (Lydon *et al.,* 1995). This is further supported as sexual behaviour can be abrogated by direct delivery of antisense PR oligonucleotides to the cerebral ventricle of the rat brain (Mani *et al.,* 1994). P4 is also known to stimulate γ-aminobutyric acid (GABA) signalling pathways in specific areas of the brain. The P4 mediated increase in GABA receptor binding sites contributes to lordosis behaviour in rats, suppression of aggressive behaviour and induction of the release of GnRH (Maggi and Perez, 1984; De Bold and Frye, 1994).

PR-B has shown to be ligand-independently activated through GnRHR signalling (An *et al.,* 2009). In this study the authors show PR phosphorylation is mediated by GnRH treatments to increase co-activator affinity and promote PRE reporter-promoter and endogenous FSHβ expression in LβT2 cells (An *et al.,* 2009). Another independent study in αT3-1 cells showed GnRH treatments resulted in the same ligand-independent activation of the PR, increasing SRC-3 interaction and luciferase expression on a PRE-luciferase reporter construct (An *et al.,* 2006).

1.6.3 Regulation of PR mRNA expression by hormones

The expression of both PR isoforms has been shown to be up-regulated in response to E2 in MCF-breast cancer cells (Milgrom *et al.,* 1973), while P4 has been shown to repress PR expression in many reproductive tissues. Furthermore, P4 exposure was able to oppose the effect of E2-mediated PR expression (Milgrom *et al.,* 1973).

In primary pituitary gonadotrope cells, PR expression was found to be up-regulated in responses to pro-longed 0.2 nM E2 treatments (Turgeon and Waring, 2006). In rat pituitary cells PR mRNA is induced by E2 and rapidly but transiently down-regulated by progesterone

(Turgeon and Waring, 2000). However in LβT2 cells, PR mRNA expression was not regulated in response to the same E2 treatments (Turgeon and Waring, 2006).

1.6.4 The membrane-bound progesterone receptor (mPR)

In 2003, a new family of progesterone receptors unrelated to nuclear receptors, but instead characteristic of GPCRs, was discovered (Zhu *et al.,* 2003; Zhu *et al.,* 2003). Non-genomic actions of progesterone have been reported in several tissue types, including the brain, kidney, intestine, testis and ovaries (Losel and Wehling, 2003). Rapid progesterone responses are also evident in cell types that lack nuclear or classical PR, such as T-lymphocytes, platelets and the rat corpus luteum (Bar *et al.,* 2000; Park-Sarge *et al.,* 1995). Examples of non-genomic actions of P4, that suggest the involvement of receptors besides the nuclear PR, include the acrosome reaction in sperm characterised by a rapid increase of intracellular Ca^{2+} (Blackmore *et al.,* 1991; Luconi *et al.,* 2004), and initiation of oocyte maturation in amphibians and fish (Maller, 2001; Thomas *et al.,* 2004).

The first α isoform of a membrane-bound progesterone receptor (mPR) was cloned from the ovary of spotted sea trout and was reported in be involved in initiating the oocyte maturation in response to P4 (Zhu *et al.,* 2003). Thereafter an additional two isoforms of mPR (mPRβ & mPRγ) were identified (Zhu *et al.,*2003). All three isoforms have been found in a number of vertebrate species including human, mouse, pig, frog, and fish (Zhu *et al.,* 2003).

The molecular structure of the membrane bound progesterone receptor (mPR) reveals GPCR like topology, having 7-transmembrane domains, an extracellular N-terminal and intracellular C-terminal domains (Figure 13) (Tang *et al.,* 2005). Yet based on amino acid sequence homology, the mPRs belong to a larger, highly conserved family of proteins termed the Progestin and AdipoQ Receptor (PAQR) family (Tang *et al.,* 2005). According to this nomenclature, mPRα, β and γ are designated PAQR 7, PAQR 8 and PAQR 5, respectively (Tang *et al.,* 2005). This conserved family of proteins include 11 mammalian members that have been termed PAQRs because its members are thought to be progesterone or adiponectin receptors. However, in this conserved family, only mPR-related receptors are found in eukaryotes, while the other two subgroups, the adiponectin- and hemosylin III-

related receptors, are found in animal, plant and fungi (Fernandes *et al.,* 2005; Thomas *et al.,* 2007).

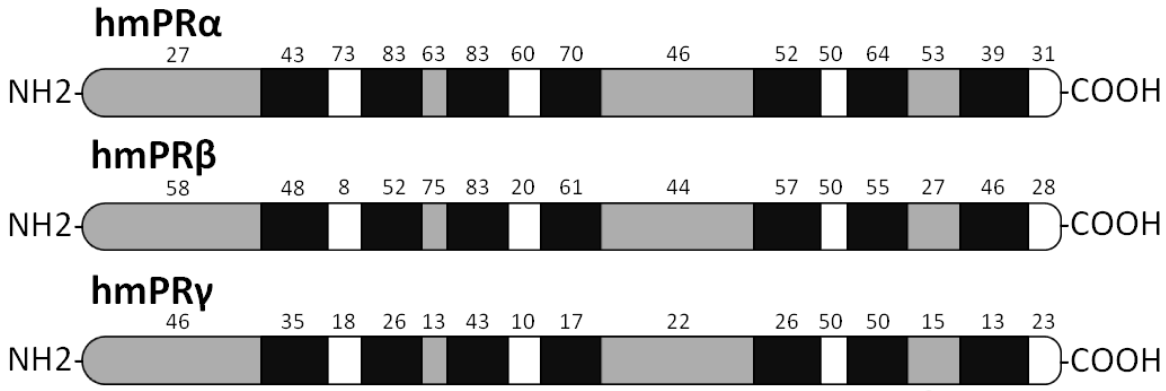

Figure 1.13: Amino acid sequence comparison between seatrout mPRα and the three putative human mPRs. Numbers above the box indicate the percentage sequence identity of amino acids in each domain between the sea trout mPR and the human mPRs. Solid gray indicate extracellular domains, solid black indicate transmembrane domains, white indicate cytoplasmic domains [addapted from Zhu *et al.,* (2003)].

The expression of each human mPR isoform appears to be tissue specific. The α-isoform is predominantly expressed in reproductive tissues including the placenta, uterus, testis and ovaries. The β isoform is exclusively expressed in neural tissues throughout the brain and spinal cord, but has not been shown in the pituitary. The γ isoform is expressed in the kidney and gastrointestinal track (Zhu *et al.,* 2003).

The binding affinities of mPRs towards P4 were initially determined using bacterially expressed recombinant mPR proteins (Zhu *et al.,* 2003), which showed specific binding of P4. An additional study supported this finding, showing that MDA-MB-231 cells transfected with human mPRα exhibit high-affinity binding for P4 (Thomas *et al.,* 2007). The synthetic progestins and antiprogestins, which have relatively high affinities for nuclear PRs, display little to no competitive binding to mPRs (Thomas *et al.,* 2007). These differences in selective binding between mPR and nuclear PR (nPR) reveal that non-genomic progesterone signalling may be exploited to design more effective drugs with fewer adverse side effects.

The link between tissues where P4 levels have an influential role, and the expression pattern of the mPRs (especially the α-isoform), suggests that mPRs may be involved in P4-dependent signal transduction *in vivo*. Zhu *et al.,* showed that expression of the sea trout mPRα in human MDA-MB-231 breast cancer cells rapidly activated the MAPK (ERK1/2) signalling cascade and lowered cAMP levels in response to P4. In addition, the effect of P4 cAMP levels were partially blocked by pre-incubations with pertussis toxin (an exotoxin which prevents G proteins from interacting with GPCRs). This suggests that the sea trout mPRα couples to an inhibitory G-protein (Zhu *et al.,* 2003). However, contradictory reports have been published regarding mPRα's ability to regulate cAMP levels through ERK1/2 activation (Kriestsch *et al.,* 2006).

The concept that mPRs couple with G-proteins has been based on a number of pertussis toxin-sensitivity assays and co-immunoprecipitation studies (Zhu *et al.,* 2003; Zhu *et al.,* 2003; Thomas *et al.,* 2007; Karteris *et al.,* 2006; Hanna *et al.,* 2006). Therefore, as putative GPCRs, mPRs may couple to a diverse array of signalling cascades, including Ca^{2+} fluxes. P4 has been reported to induce rapid Ca^{2+}mobilization in different target cells, including myocytes and sperm (Thompson *et al.,* 2004; Blackmore *et al.,* 1991). In support of this notion, the over-expression of ovine mPRα in CHO cells revealed a P4-dependent release of Ca^{2+} from the endoplasmic reticulum (Ashley *et al.,* 2006).

Therefore, as putative GPCRs, mPRs can be considered as novel pharmaceutical targets, which could lead to the design of more specific and effective drugs in controlling contraception and further reproductive disorders. However, much scepticism has been reported in the literature concerning the functional and mechanistic role of mPRs in mediating rapid P4 responses.

1.7 The glucocorticoid receptor

Glucocorticoids play a vital role in inducing many biological responses involved in growth, reproduction, metabolism, immune and inflammatory reactions, as well as central nervous system and cardiovascular function (Tilbrook *et al.,* 2000; Zhou and Cidlowski. 2005). Glucocorticoids regulate carbohydrate, protein and fat metabolism, and play a vital role in the protection of glucose-dependent tissues such as the brain and heart during stressful situations (Katzung, 2004; Goodman *et al.,* 2006). Immune responses are also modulated through GR action; regulating the activity of peripheral leukocytes, suppressing the production of cytokines and chemokines, and changing the life span of immune cells (Lu *et al.,* 2006). Therefore glucocoriticoids represent one of the most widely used therapies for many immune and inflammatory diseases including acute and chronic asthma, rheumatoid arthritis as well as in cancer treatment (Rhen and Cidlowski, 2005).

The endogenous ligand to the GR in humans is cortisol. The synthesis and secretion of glucocorticoids by the adrenal cortex is tightly regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.3). Like all HP-endocrine signalling, this axis is sensitive to negative feedback through circulating steroid hormones and glucocorticoids (Lu *et al.,* 2006).

1.7.1 The GR gene and promoter structure

The human GR (hGR) cDNA was first isolated by cloning in 1985 (Hollenburg *et al.,* 1985). The hGR gene consists of 9 exons and is located on chromosome 5. At least three separate promoters have been identified for the mouse GR gene (Figure 1.14) (Strahle *et al.,* 1992). The utilization of these separate promoters gives rise to five separate transcripts, encoding different 5'-untranslated first exons (Figure 1.14). The proximal GR-promoter region (1B and 1 C) is very GC-rich, contains a CpG island characteristic of housekeeping genes and lacks TATA boxes and CAAT boxes, which is consistent with the fact that the GR is ubiquitously expressed (Breslin *et al.,* 2001). There are four Sp-1 sites upstream to the exon 1B transcriptional start site. Transcriptional regulation through GC-rich regions is complicated, as several factors other than Sp-1 can recognise this sequence (Berg, 1992). Tissue-specific alternative splicing has been shown on exon 1A (Figure 1.14 B). Transcripts 1A1 and 1A2

share a common promoter with transcript 1A3, yet they have a broader expression pattern (Breslin *et al.,* 2001).

Computer analysis has suggested that there is a half-GRE present in the 1C promoter region, and that this sequence can bind GRβ. However deletion studies did not completely abolish Dex responsiveness. Finally a site resembling the nuclear factor-κB (NF-κB) site was found on the 1C promoter region, with preliminary studies showing NF-κB can bind to this site (Breslin *et al.,* 2001).

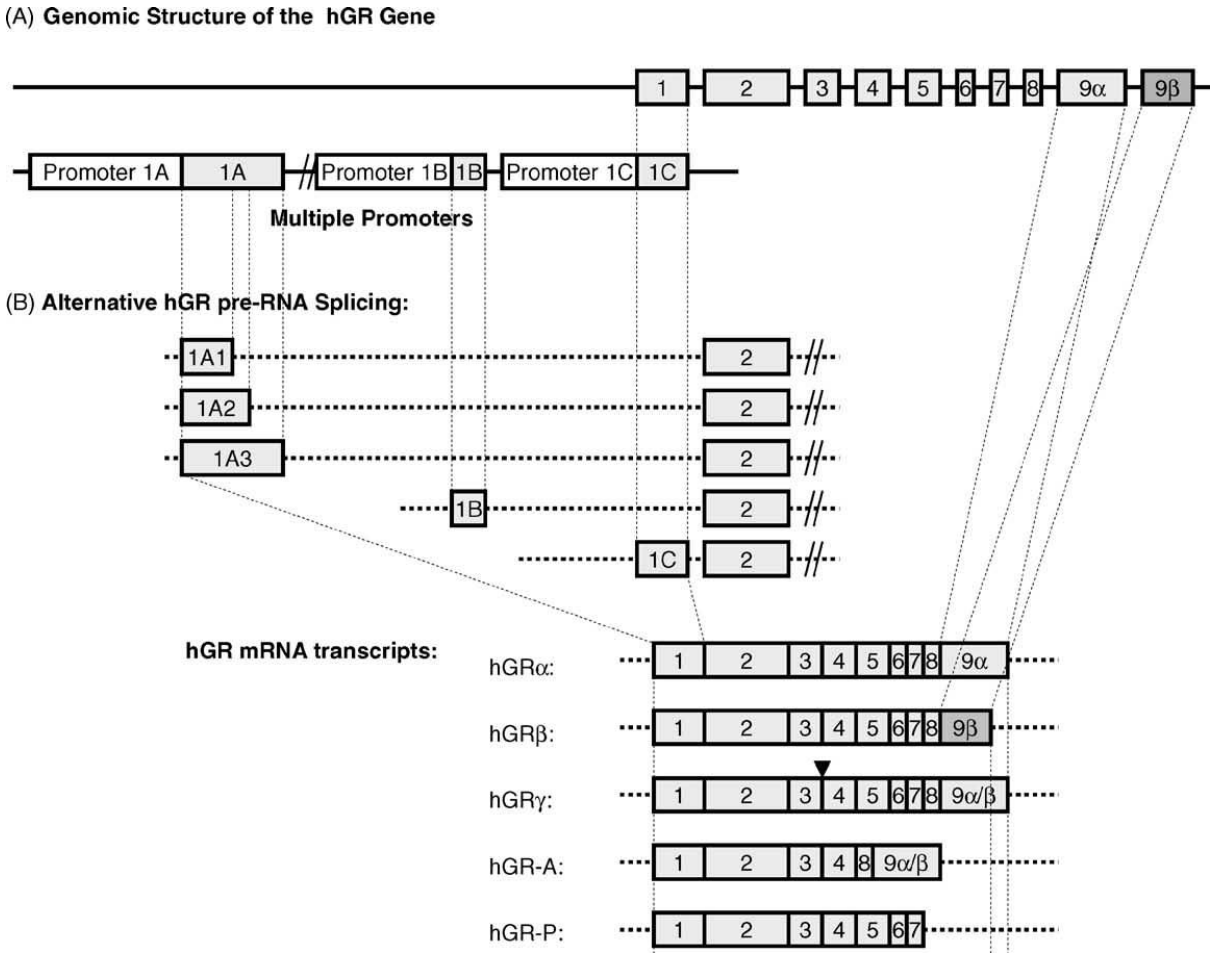

Figure 1.14 (A-B): Generation of multiple hGR isoforms form a single gene. (A) Genomic structure of the hGR gene. (B) The generation of multiple hGR transcripts as a result of alternative RNA splicing (Zhou and Cidlowski, 2005).

Alternative splicing of the hGR gene in exon 9 generates two highly homologous receptor isoforms, GRα and GRβ (Figure 1.14) , which are 777 and 742 amino acid in length, and 97

and 94 kDa in molecular weight, respectively (Figure 1.15) (Zhou *et al.,* 2005; Cidlowski, *et al.* 1990; Oakely, *et al*. 1996).

These two isoforms have identical N-terminal domains, and differential C-terminal domains (Figure 1.15) (Lu and Cidlowski, 2005). hGRα resides in the cytoplasm and represents the classical GR that functions as a ligand-dependent transcription factor. hGRβ exerts a dominant negative effect on the transcriptional activity of hGRα, and shows cell specific differences in transcriptional activity compared to hGRα in response to Dex (Oakley *et al.,* 1999; Kino *et al.,* 2009).

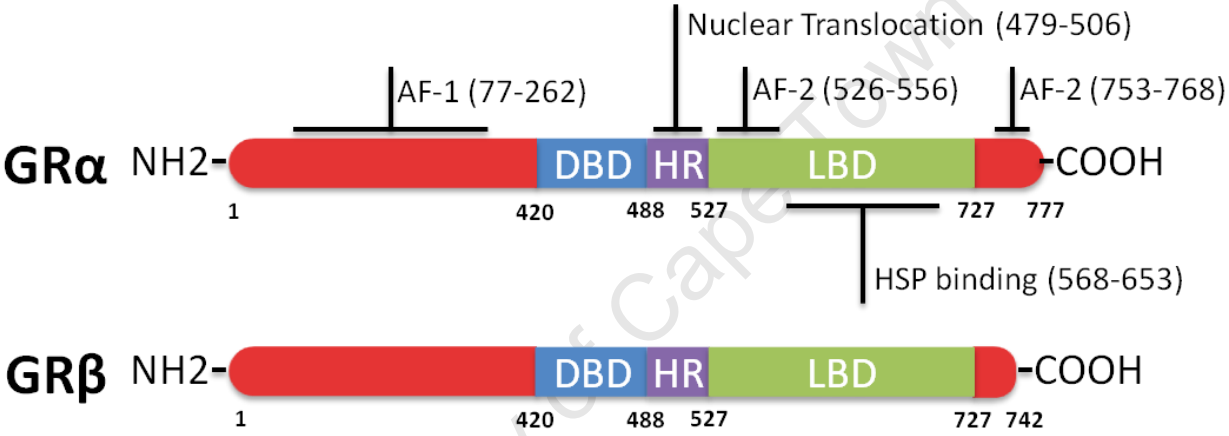

Figure 1.15: Primary structure comparison between the glucocorticoid receptor isoforms α and β. LBD (Ligand binding domain); DBD (DNA binding domain); HR (hinge region); and AF (transcriptional activation domains 1 and 2) are shown. Illustration shows comparison between GRα and β GR isoforms. The amino acid positions for each domain are indicated under primary structure. The positions of additional sub-domains are indicated for the GRα isoform. Illustration not to scale [adapted from Nicolaides *et al.,* (2010)].

1.7.2 GR-mediated signalling

In the absence of ligand hGRα resides in the cytoplasm in a hetero-oligomeric complex containing heat shock proteins (HSP) 90, 70 and 50, immunophilins, as well as other proteins (Pratt *et al.,* 1993). HSP 90 exposes the ligand domain and mediates ligand binding, while retaining cytoplasmic location through masking the nuclear localisation sequence (Figure 1.14) (Terry *et al.,* 2007). Immunophilin proteins are thought to be involved in GR nucleo-cytoplasmic shuttling, as a study has shown immunophilins FKBP51 and FKBP52 interact with the hGRα to mediate cytoplasmic or nuclear localisation, respectively (Davies *et al.,* 2002; Davies *et al.,* 2005; Echeverria *et al.,* 2009).

Upon ligand-induced activation, GRα undergoes a conformational change that results in dissociation from the multi-HSP protein complex and translocates to the nucleus (Pratt *et al.,* 1993). To initiate transactivation, the GRα homodimer binds to an inverted hexametric palindromic DNA sequence, and interacts with receptor co-activators and chromatin remodelling complexes as described in Section 3.2. Some GC-responsive genes containing simple-acting GREs include the serine/threonine protein kinase, tyrosine hydroxylase and tyrosine aminotransferase (TAT) genes (Schoneveld *et al.,* 2004; Schmid *et al.,* 1987).

Alternatively ligand bound GRα can modulate gene expression independent of GRE binding, through GR monomer and NF-κB, AP-1 or STAT protein-protein interactions (Kassel and Herrlich. 2007). The effects of glucocorticoids on the immune system are mediated by the transrepressive effects on pro-inflammatory transcription factors AP-1 and NFκB. GRα inhibits AP-1 and NF-κB mediated transcription through interacting with, and blocking subsequent transcription initiation complex formation (Jonat *et al.,* 1990; Mukaida *et al.,* 1994). In addition to transrepression, GR protein-protein interactions can also lead to a synergistic induction of promoter activity, as seen with the interaction of GR and Stat-5 on the β-casein promoter (Stocklin *et al.,* 1996).

Post-translational modifications have been shown to affect GR signalling (Avenant, *et al* 2010; Nicolaides *et al.,* 2010). The hGRα has several phosphorylation sites including serines at positions 113, 141, 203, 211, 226 and 404, located in the AF-1 sub-domain. Phosphorylation usually occurs after ligand binding, and modulates GR-mediated

transcriptional activity, sub-cellular trafficking, target promoter specificity, duration of GR mediated signalling, and GR stability (Ismaili and Garabedian. 2004). However, phosphorylation at Ser 203, 211 and 226 does not affect angonist-induced hGR degradation, but has been shown to regulate GR-mediated transactivation through co-factor recruitment (Avenant *et al.,* 2010)

The GR is also modulated through acetylation, which occurs after ligand-binding and prior to nuclear translocation (Ito *et al.,* 2006). A known acetylation site of the GR is the leucine rich region in the hinge domain (amino acids 492-465, sequence KKTK), which is analogous to the acetylation sites found in other SRs. Mutations at K494 and 495 on the hGR that prevented acetylation reduced the sensitivity to Dex suppression of interleukin 1beta-induced granulocyte/macrophage colony-stimulating factor production, however, did not affect the repression of NF-κB genes (Ito *et al.,* 2006). An additional study has shown that the circadian rhythm-generating transcription factors CLOCK and BMALI repress GR-induced transcriptional activity by acetylating several lysine residues located in the hinge region. This post-translational modification attenuates GR binding to GREs, and its ability to glucocorticoid-responsive gene expression (Nader *et al.,* 2009).

1.7.3 Regulation of GR mRNA expression by hormones

GR mRNA expression is regulated by glucocorticoids. However, no full consensus GRE, TATA-box or CAAT-box can be found in the promoter region or in the first untranslated exon of the hGRα gene (Zong *et al.,* 1990). A study has shown that cells which contain endogenous GR protein undergo down-regulation in GR protein GC-binding capabilities and GR mRNA expression (Burnstein *et al.,* 1990). This study suggests that GR mRNA and protein are down-regulated in responses to GCs (Burnstein *et al.,* 1990).

In most cells tested to date, GR mRNA and protein are down-regulated by glucocorticoids, and has been extensively proven in several cell lines including the HeLa cell line (Burnstein *et al.,* 1990; De Silva *et al.,* 1993; Freeman *et al.,* 2004). A representation of this model is shown in the HPA signalling axis (Figure 1.3). An exeption to the suppressive role of glucocorticoids are T-lymphocytes, thymocytes and T-lymphoblasts, as glucocorticoid

treatment results in the up-regulation of GR mRNA expression to aid in subsequent apoptosis (Breslin *et al.,* 2001; Burnstein *et al.,* 1990; Ramdas *et al.,* 1999).

1.8 Extra-nuclear signalling by steroid receptors

The first evidence for rapid steroid signalling at the plasma membrane was published in 1967, showing that an E2 binding protein at the plasma membrane of cells induced a rapid stimulation of cAMP production and Ca^{2+} flux in the uterus of rodents (Szego and Davis, 1967). In various cells and tissue types a large variety of cytoplasmic signalling pathways have since been reported to be activated in response to steroid treatment. Depending on the steroid hormone and tissue-specific response, it appears that different receptors and mechanisms are involved in mediating the rapid actions of steroids. In the literature, four such mechanisms have been proposed to mediate these rapid effects of steroid hormones (Edwards, 2004).

The first mechanism involves novel membrane receptors that are unrelated to conventional SRs, yet have been shown to mediate the specific non-genomic actions of hormones. The first protein/receptor that fulfils the requirements of a novel membrane receptor for P4 is mPR (Thomas *et al.,* 2007). This receptor was cloned and characterised as a GPCR involved in mediating P4-induced oocte maturation in seat trout (Zhu *et al.,* 2003) (Refer to 5.1).

The second mechanism suggests that classical SRs interact with or are modified by signalling responses at the plasma membrane through GPCR crosstalk. Support for this is seen in the case of ER signalling in CHO cells. The E2 induced rapid stimulation of adenyl cyclase activity and inositol phosphate (IP3) formation was associated with the involvement of ERα and the activation of $G_{\alpha S}$ & $G_{\alpha q}$ proteins (Razandi *et al.,* 1999). Additionally, rapid phosphorylation of the unliganded PR and GR was shown to be mediated by the GnRHR in the LβT2 cell line, to regulate downstream target gene expression (An *et al.,* 2009; Kotitschke *et al.,* 2009).

The third mechanism suggests that sub-cellular location of SRs is important in modulating the activities of extra-nuclear cell signalling pathways. Here plasma membrane association is

the key for mediating the rapid effects of steroid hormones. Several truncated SRs have been identified that are linked to the rapid actions of hormones. Post-translational modifications of these isoforms have been shown to promote cell membrane association (Li *et al.,* 2003). These truncated SR isoforms have been involved in mediating many rapid signalling responses in a hormone-dependent manner, including the activation of MAPK pathways in the Neo-cortex (Toran-Allerand *et al.,* 2002; Marquez and Pietas, 2001), activation of PKC and Src in oesteoblasts (Longo *et al.,* 2004), and the induction of Ca^{2+} fluxes in ovarian granulosa cells (Peluso *et al.,* 2002; Peluso *et al.,* 2003). It is thought that truncated SRs, through altered folding properties, are better substrates for lipid modifications and differential cell compartmentalization compared to full length SRs.

The fourth and final mechanism suggests a subpopulation of conventional SRs associate with signalling complexes in the cytoplasm or the plasma membrane to induce the non-genomic actions of steroid hormones. Several studies have shown that SR plasma membrane localisation is facilitated by the association of scaffolding proteins that translocate the protein-SR complex to the cell membrane. These scaffolding proteins include caveolin-1, Shc and insulin-like growth factor 1 (IGF1). Caveolin-1 is a major structural protein of caveolae (present in plasma membrane micro domains), and has been shown to physically interact with the ER in endothelial cells (Razandi *et al.,* 2002), and promote ER translocation to the plasma membrane (Razandi *et al.,* 2002). Another adaptor protein Shc, complexes with the ER to promote plasma membrane translocation, where it associates with IGF1 receptor in an E2 dependent manner in MCF-7 breast cancer cells (Song *et al.,* 2004).

One of the better characterised signalling actions of steroids is the rapid activation of the Src/ras/raf/MAP kinase (Erk) pathway. Through co-immunoprecipertation and pull down assays, it has been shown that the ER and PR interact with Src in a ligand-dependent maner (Song *et al.,* 2002; Wong *et al.,* 2002; Edwards *et al.,* 2002; Migliaccio *et al.,* 1998). Src is a key component in the coupling of extracellular signals with a variety of intracellular signalling transduction pathways, with SR-mediated Src/MAPK activation involved in multiple cell functions including proliferation, differentiation and apoptosis (Martin, 2001; Thomas and Brugge, 1997).

ER and PR interact with Src though a 120 kDa adaptor protein termed modulator of nongenominc action of estrogen Receptor (MNAR). The N-terminal domain of MNAR contains multiple LxxLL motifs that interact with the AF-2 domain of the ER (Heery *et al.,* 1997) and a short polyproline sequence (PPPLPPR) of the PR (Figure 1.12) (Boonyaratanakornkit *et al.,* 2001). Three PxxP motifs on MNAR have been shown to interact with the SH3 domain of Src (Barletta *et al.,* 2004). These protein-protein interactions are responsible for stabilizing the Scr/ER or PR complex. Thus, the LxxLL motifs of MNAR may also interact with other SRs including the AR, GR and PR, in a ligand-dependent manner (Barletta *et al.,* 2004; Wong *et al.,* 2002). However, this mechanism has not been investigated for other SRs in mediating extra-nuclear signalling.

1.9 Integration of nuclear and extra-nuclear signalling pathways of steroid receptors to regulate gene expression

It has become increasingly more evident that conventional SRs are multi-functional proteins, capable of acting in the nucleus as ligand-dependent transcription factors and outside the nucleus to activate non-genomic signalling pathways. There is a body of knowledge regarding the conventional ligand-dependent mechanisms of SR-medaited gene regulation (Avenant, 2009; Weigel and Moore, 2007) (Figure 1.16 - pathway 1). This pathway shows SR-mediated gene regulation via ligand-dependent HRE binding and/or tethering mechanisms (refer to Section 1.4.2).

The section above highlights the role SRs have in initiating extra-nuclear signalling pathways. SRs regulate extra-nuclear signalling cascades through interacting with scafolding proteins and signalling complexes at the cell membrane (Song *et al.,* 2002; Wong *et al.,* 2002; Edwards *et al.,* 2002). Alternately the extra-nuclear effects of steroids can be mediated by putative mSRs to initiate extra-nuclear signalling cascades (Figure 1.16 – 2).

Many extra-nuclear signalling pathways converge to activate nuclear transcription factors through post-translational modifications. This suggests that SR-mediated extra-nuclear signalling may ultimately affect gene expression patterns within the cell. Therefore to the knowledge of the author, the rapid steroid-induced activation of cell signalling events can influence the outcome of steroid-mediated gene expression through several hypothesised mechanisms, which have been represented in Figure 1.16.

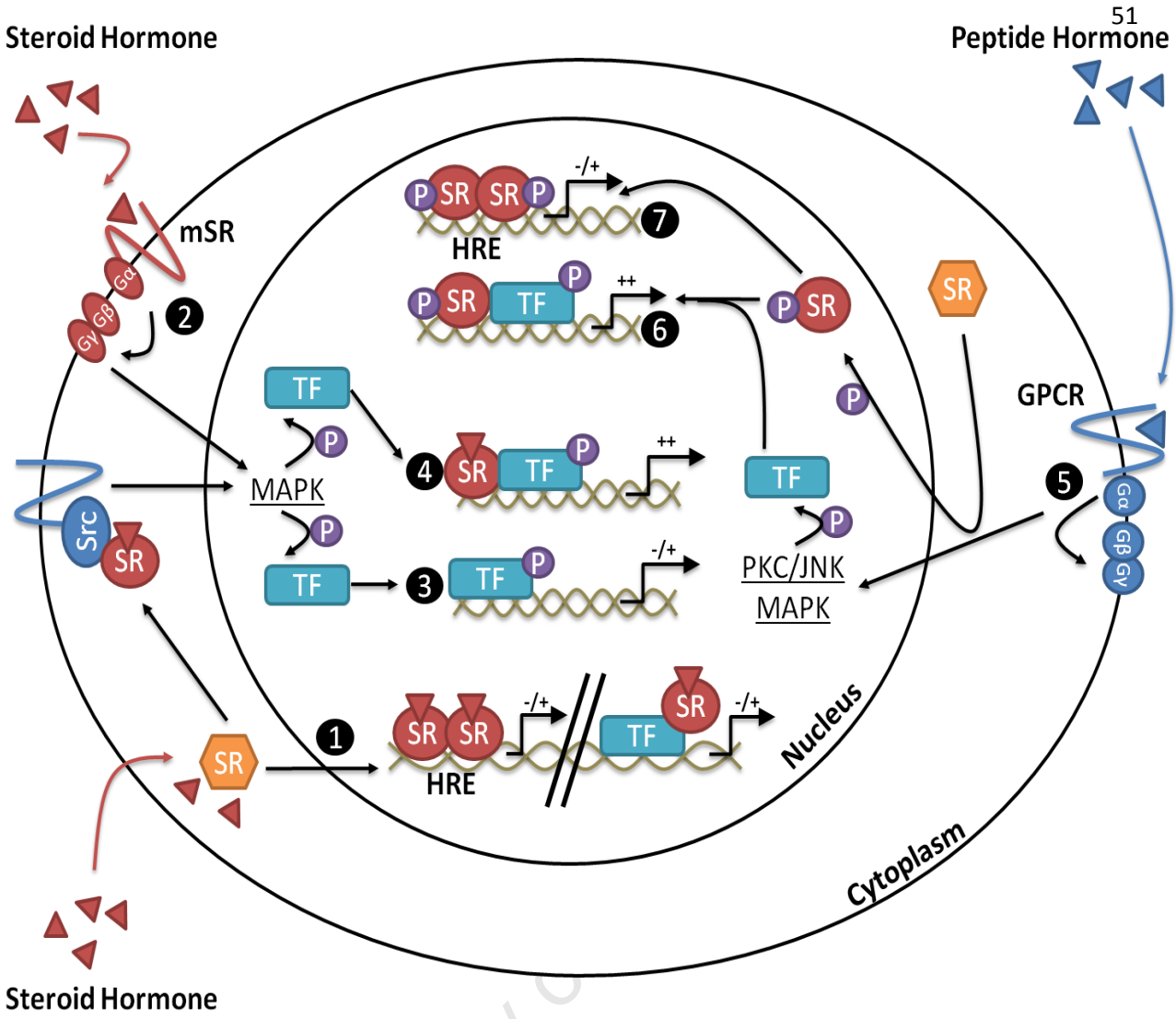

Figure 1.16: Intergration of nuclear transcription and extranuclear signalling by conventional SRs and GPCRs. All numbered pathways are relavent to this current sIn the classical nuclear transcription pathway (1); steroid hormones activate steroid receptors (SR) by inducing conformational changes to promote nuclear translocation, dimerization, and direcet or indirect binding to promoters of target genes. Activated SR bound to target promoter DNA can regulate (positively or negatively) target gene transcription via HRE or tethering mediated mechanisms. A subpopulation of steroid receptors can associate with cytoplasmic or cell membrane signalling molecules including the tyrosine kinase Src in a hormone-dependent manner. This interaction leads to an activation of Src and the downstream MAPK signalling cascades (not numbered on figure). In addition to this, novel membrane receptors (mSR), unrelated to conventional receptors, have heen shown to activate MAPK signalling (2). Therefore steroid-induced activation of MAPK may influence gene transcription by 2 hypothesised mechanisms. The first hypothesis is genes that lack HREs can be regulated in response to steroid hormones (3). The second hypothesis is that HRE-regulated genes can be influenced bytranscription factors (TF), whose activities are regulated by MAPK signalling (4). Two additional pathways have been hypothesised to regulate target gene expression via SR and GPCR

crosstalk signalling (5). The first hypothesis is that GPCR ligands can ligand-independently activate SRs to regulate SR- and GnRHR-target gene expression via SR/GPCR crosstalk (6).The second hypothesis is that GPCR ligands can induce the expression of HRE regulated genes through ligand-independent activation of SRs via by SR/GPCR crosstalk (7). (mSR – membrane steroid receptor; HRE – hormone response element; TF – transcription factor; SR – steroid receptor; P – phosphorylation; GPCR – G-protein coupled receptor) [figure adapted from Edwards, (2006), Schoneveld *et al.,* (2004) and Kotitschke *et al.,* (2009)].

The first potential hypothesised mechanism suggests target genes that lack conventional HREs, may be regulated in response to steroids (Figure 1.16 – pathway 3). This may be mediated by the liganded SR interacting with a signalling complex at the cell membrane (not shown on Figure 1.16) or via putative mSR signalling (Figure 1.16 – 2), to activate MAPK signalling and subsequent transcription factors (TFs) (Figure 1.16 – pathway 3). This was shown to be the case in the MCF-7 breast cancer cells, where E2-induced c-*fos* mRNA expression is mediated by a serum response element (SRE) in the proximal promoter, and not an ERE. Furthermore *c-fos* mRNA expression was mediated by an ER-dependent activation of the Src/Ras/MAPK pathway to activate the Elk1 transcription factor (which can bind to and transactivate an SRE) (Duan *et al.,* 2001); or the Src-RAS/PI3K pathway to activate the serum response factor (SRF) (which can bind to and transactivate an SRE) (Duan *et al.,* 2002). This study helps supports the hypothesis that steroid-induced MAPK signalling can regulate target gene promoters that lack HREs, further expanding the gene "network" of steroid hormones.

The second mechanism proposes that complex promoters of SR target genes contain additional binding sites for other transcription factors, suggesting a required cooperative interaction between transcription factors and SRs. This cooperation may occur through post-translational modifications of transcription factors, mediated by protein kinase cascades (which are activated by SRs) (Figure 1.16 – pathway 4). This hypothesis is supported by a study from Watters *et al.,* (2001) who show liganded ER directly interacts with EREs in the promoter region of the prolactin gene to positively influence gene expression, but is also dependent on an acute E2-dependent ER-mediated activation of the

MAPK pathway (Walters *et al.,* 2001). This suggests that E2 induction requires cooperative interactions between the ER (bound to EREs) and other transcription factors modulated by MAPK signalling to enhance gene expression (Vasudevan *et al.,* 2001). Furthermore the effects of GR binding to a GRE in the proliferin gene can either result in transactivation or transrepression, depending on the composition of the AP-1 proteins, c-jun and c-fos, on an adjacent AP-1 site (Miner and Yamamoto, 1992; Pearce *et al.,* 1998). These studies confirm the hypothesis that target HRE-regulated promoters can be influenced by other transcription factors, whose activities are regulated through MAPK signalling (Figure 1.16 – pathway 4).

This integration of nuclear and extra-nuclear signalling mediated by SRs, is what makes crosstalk signalling between endocrine signalling axes achievable. Therefore, these hypothesised mechanisms outlined above, give some insight into how target mRNA levels may be regulated in response to hormones treatments. It must be noted that the involvement of signalling complexes at the cell membrane (including as Src) in mediating steroid-induced responses will not be assessed in this study. Instead emphasis will be laid on the involvement of putative mSRs.

1.9.1 Integration of nuclear and extra-nuclear signalling between GnRHR and other SRs in pituitary gonadotropes

In the past, the mechanisms of action of a particular SR have usually been studied in isolation. It is becoming increasing clear that endocrine signalling is not one-dimensional. However different signalling pathways must crosstalk in order to fine tune and integrate the cell's responses to multiple and simultaneous signals from the environment. Steroids orchestrate a broad and complex array of signalling cascades beyond the classical genomic role of SR-dependent gene transcription. Considering that all SRs act via similar mechanisms, insights into the function of one is likely to be relevant to the group of SRs as a whole.

Recent studies are beginning to reveal crosstalk mechanisms between the SRs and GnRHRs that mediate a number of HPG signalling events at the level of gonadotropes (An *et al.,* 2009; Kotitschke *et al.,* 2009; Chen *et al.,* 2009) (Figure 1.16 – 5). These studies were performed in the LβT2 cell line, and represent important findings with regards to the ligand-independent activation of SRs mediating gonadotrope cell responses.

The study performed by An *et al.,* (2009) revealed a crosstalk mechanism between the PR and GnRHR (Figure 1.16 – pathway 6). Here they demonstrated that GnRH treatments induce PR phosphorylation to increase its interaction with the co-activator nuclear co-activator 3 (NCoA3). This enhances PR recruitment to the PRE-region of the fshβ promoter through co-factor interaction. An *et al.,* further shows PR-B is phosphorylated at Ser-249 in response to GnRH by PKC, promoting interaction with the co-activator NCOA3. It is this co-factor recruitment that results in the rapid and positive effect GnRH has on fshβ expression in the LβT2 cell line (An *et al.,* 2009).

A study performed by Kotitschke *et al.,* (2009) revealed a crosstalk mechanism between the GR and GnRHR. The study showed that Dex and GnRH act synergistically to regulate *GnRHR* expression in LβT2 cells in a GR-dependent manner (Figure 1.16 – pathway 6). Kotitschke *et al.,* showed that Dex treatment resulted in the Dex-mediated activation of the GR (Figure 1.16 – pathway 1), while GnRH treatment resulted both the activation of c-Jun/c-fos transcription factors, and the ligand-independent activation of the GR through site specific post-translational modifications (Figure 1.16 – pathway 6). Furthermore the ligand-

independent effects of GnRH on the GR were replicated on a TAT-GRE-Luc reporter in LβT2 cells (Figure 1.16 – pathway 7) (Kotitschke *et al.,* 2009).

A study performed by Chen *et al.,* (2009) revealed that GnRHR and ERα crosstalk to transcriptionally regulate the fosB gene in the LβT2 cell line. The data suggests that GnRH-mediated phosphorylation of ERα results in the rapid association with the co-factor PCAF. This increases the co-recruitment of the ERα to an ERE within the endogenous *fos*β promoter region in LβT2 cells, in turn activating *fos*β expression (Figure 1.16 – pathway 6). The authors also demonstrated that this mechanism activates other genes in the LβT2 cell line, including the fshβ sub-unit gene (Chen *et al.,* 2009).

Taken together these studies show signalling pathways mediating the actions of GnRH and steroid hormones are dependent on SRs, and converge to regulate gene expression in gonadotrope cells. Furthermore, nuclear and extra-nuclear signalling is likely to reveal further insight into the mechanisms underscoring important biological functions, including the neurological, immune, stress, and reproductive signalling axes. Finally, putative mechanisms for signalling crosstalk between hormone receptors will give a new perspective on possible drug targets, and generate a better understanding of the endocrine signalling system at a biochemical level.

CHAPTER 2

MATERIALS AND METHODS

2.1 Compounds and antibodies

Dex, P4, E2, GnRH and the P4 agonist, R5020, were purchased from Sigma-Aldrich, South Africa. Polycolonal antibodies for the mammalian GR (H-300, sc-8992), PR-B (C-20, sc-539), ERα (MC-20, sc-542) and the HA-tag (Y-11, sc-805), as well as the monoclonal antibodies for the mPRα (Y-14, sc-50113), the human PR-B (B-30)(sc-811) and PR(A+B) (AB-52) (sc-810), were obtained from Santa Cruz Biotechnology, USA. Polycolonal antibodies for Flotilin-1 (#610820) were obtained from BD Biosciences, USA. Polyclonal antibodies for p44/42 MAP Kinase (ERK1 and ERK2) (#9102) were obtained from Cell Signalling, South Africa. The monoclonal antibody for β-Tubilin (T4026) was obtained from Sigma-Aldrich, South Africa. The secondary anti-rabbit HRP conjugate (sc-2313), anti-mouse HRP conjugate (sc-2005) and anti-goat HRP conjugate (sc-2350) antibodies were purchased from Sigma-Aldrich, South Africa. Refer to section 2.11 for dilutions used for each specific primary antibody, as well as the corresponding secondary antibody and dilution thereof.

2.2 Incubation with test compounds

Dex, P4, and R5020 were diluted in 100% EtOH to a final concentration of 100 µM. E2 was diluted in 100% EtOH to a final concentration of 100 nM. GnRH was purchased as a lyophilized salt and dissolved in H_2O to a final concentration of 100 µM. All test compounds were added to cells as 1/1000 dilution in culture medium.

2.3 Plasmids

Wild type HA-tagged human GR (pCMV-HA-human GR) expression vector was obtained from Prof. M.J. Garabedian at NYU Department of Microbiology, New York. The pTAT-GRE-E1b-luc plasmid was a gift from Dr G. Jenster at Erasmus University of Rotterdam, Netherlands and the pCMV-ß-galactosidase plasmid was a gift from Dr. G. Haegeman (University of Gent, Belgium). The pMT-PR-B was gift from Prof. S. Okret (Karolinka Institute, Sweden). The expression plasmid pSG5-hERα was a gift from F. Gannon (EMBL, Germany) and the ERE.vit2-luc was a kind gift from K. Korach (National Institute of Environmental Health Science, USA). The human mPRα expression plasmids (pcDNA3.1/hmPRα, pcDNA3.1/V5/hmPRα and pHA/hmPRα) were kindly given from Dr. B. Gellersen (Endokrinologikum Hamburg, Germany). Plasmid maps were generated for mPRα constructs through DNA sequence analysis (Figure 12.1, Figure 12.2 and Figure 12.3).

2.4 Cell culture

LβT2 mouse pituitary gonadotrope cells were kindly provided by Dr. P. Mellon from the University of California, San Diego, USA. COS-7 monkey kidney fibroblast cells were purchased from American Type Culture Collection (ATCC, USA). Both cell lines were maintained in high glucose Dulbeccos's Modified Eagles Medium (DMEM, Sigma-Aldrich, South Africa) supplemented with 10% fetal calf serum (FCS) (Delta Bioproducts, South Africa), 50 µg/ml penicillin and 50 U/ml streptomycin (Gibco-BRL Life Technologies, UK), unless stated otherwise. All cultures were maintained in 75 cm^2 culture flasks (Greiner Bio-one International, Austria) at 37°C, in an atmosphere of 90% humidity and 5% CO^2. LßT2 cells and COS-7 cells were passaged with 0.25% trypsin / 0.1% EDTA in calcium- and magnesium-free PBS (Highveld Biologicals, South Africa) once or twice a week, respectively. LßT2 cells were very sensitive to over-trypsinising, and were therefore not incubated in the trypsin solution for longer than 3 minutes. All cell lines were regularly tested for mycoplasma infection by means of Hoechst staining (Freshney, 1987), with only mycoplasma-negative cells used.

End1/E6E7 (endocervical cells immortalised with the human papillomavirus 16/E6E7) cells were purchased from the American Type Culture Collection (ATCC), United States of

America. The End1 cells were gown in Stemline™ Keratinocyte Medium II (KSF) (Sigma-Aldrich, South Africa) supplemented with $CaCl_2$ (final concentration 0.4 nM), 100 U/µl penicillin and 100 µg/µl streptomycin (Gibco-BRL Life Technologies, UK), as were the bovine pituitary extract (BPE) Stemline™ Keratinocyte Growth Supplement, Sigma-Aldrich, South Africa). Passaging and maintenance of the Endo-cervical cell line was performed by Nicky Verhoog. (Verhoog, 2010)

2.5 Plasmid transformation and preparation

The plasmids were transformed into *Escherichia coli* DH5α cells by heat shock transformations according to Sambrook *et al.* (Sambrook *et al.,* 1989). Briefly, 100 µl competent cells were mixed with 10 ng of DNA. The mixture was placed on ice for 30 min, followed by 2 min incubation at 42°C and 2 min incubation on ice. Immediately after transformation, cells were mixed with 900 µl SOC medium [2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose] (Sambrook *et al.,* 1989) and incubated (1 hour, 37°C while shaking at 200 rpm). Cells were subsequently plated out on LB agar plates [1% (w/v) tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar containing 50 µg ampicillin] (Sigma-Aldrich, South Africa) and allowed to grow overnight at 37°C (Sambrook *et al.,* 1989). The following day, colonies were picked and grown in 50 ml LB medium [1% (w/v) tryptone, 0.5% yeast extract and 1% NaCl, containing 50 µg/ml ampicillin] for at least 8 hours at 37°C while shaking. For glycerol stocks 500 µl 80% (w/v) glycerol was mixed with 500 µl of the cell suspension and stored at -80°C. For plasmid preparations, LB medium containing 50 µg/ml ampicillin was inoculated and allowed to grow overnight at 37°C while shaking. The next day the plasmid DNA was purified with the GeneElute™ Plasmid Midiprep Kit from Sigma (Sigma-Aldrich, South Africa), according to the manufacturer's protocol. The integrity and purity of the plasmids were tested by restriction digestion and agarose gel electrophoresis.

2.6 Transient transfections and reporter assays

LßT2 cells were plated in 12-well culture plates (Greiner Bio-one, International, Austria) at a density of 1 X 10^5 cells per well in 1 ml DMEM supplemented with 10% FCS and antibiotics as described elsewhere. Twenty-four hours after plating, the medium was replaced with phenol red-free Dulbecco's Modified Eagles Medium (D1145) (Sigma-Aldrich, South Africa) supplemented with 10% charcoal-striped FCS serum (#306) (Highveld Biological, South Africa) and antibiotics (as describedin Section 2.4). Cells were transfected with 250 ng pTAT-GRE-E1b-luc or 300 ng ERE.vit2-luc promoter-reporter constructs. In order to correct for transfection efficiency, 25 ng pCMV-ß-galactosidase was co-transfected. All transfections were performed according to the FuGENE^{TM} 6 (Roche Applied Science, South Africa) product protocol, at a ratio of 1µg DNA: 2 µl FuGENE^{TM} 6. At least 24 h after transfection, the medium was replaced with phenol red-free Dulbecco's Modified Eagles Medium (D1145) (Sigma-Aldrich, South Africa), serum-free medium containing antibiotics (described in Section 2.4). Incubations were performed with test compounds as indicated in appropriate figure legends. For reporter assays, cells were harvested in 50 µl reporter lysis buffer (Promega Corp., USA) per well. The luciferase assays (Luciferase Assay System, Promega, USA) and β-galactosidase assays (Galacto-Star, Tropix Inc, USA) were carried out with 10 µL cell extract in white 96-well plates in a Modulus microplate reader (Turner Biosystems, USA). Luciferase values were normalised to corresponding β-galactosidase values and expressed relative to vehicle control values.

2.7 Isolation of total RNA

LβT2 cells were plated in 12-well culture plates (Greiner Bio-one, International, Austria) at a density of 4,5 X 10^5 cells per well in 1 ml DMEM medium supplemented with 10% FCS and antibiotics (as described Section 2.4). Twenty-four hours after plating, the medium was replaced with Phenol red-free Dulbecco's Modified Eagles Medium (D1145) (Sigma-Aldrich, South Africa) supplemented with 10% charcoal stripped serum and antibiotics (as described in Section 2.4). If indicated, a concentration of 0.2 nM E2 was added to each sample as shown in appropriacte figure legends. Seventy-two hours after plating, the medium was replaced with phenol red-free Dulbecco's Modified Eagles Medium (D1145) (Sigma-Aldrich,

South Africa), serum-free media, containing antibiotics (described in Section 2.4) and incubated with 100 nM test compound for 8 hours, as indicated in the figure legends. Total RNA was isolated with Trizol reagent (Sigma-Aldrich, South Africa) according to the manufacturer's protocol. Briefly, 400 µl Trizol reagent was added per well and incubated at RT for 5 min. Thereafter cell lysates were transferred into microcentrifuge tubes and centrifuged for 10 min at 12 000 g (4°C). The supernatant was transferred into new microcentrifuge tubes, 80 µl chloroform was added to each sample and vortexed for 15 sec. Thereafter the samples were centrifuged for 15 min at 14 000g (4°C). The top aqueous phase (125 µl) containing the RNA was transferred into new microcentrifuge tubes and 200 µl of isopropanol was added. Subsequently, the samples were incubated at RT for 10 min followed by centrifugation for 10 min at 14 000 g to pellet the RNA (4°C). The RNA precipitates were washed twice with 400 µl 70% EtOH and centrifuged for 5 min at 14 000 g (4°C). The RNA was allowed to air dry for 5 min and re-suspended in 15 µl DEPC-treated-water (Addendum F). The RNA was then incubated at 55°C for 5 min, where after the samples were stored at -20°C.

To confirm the integrity of the isolated RNA, denaturing formaldehyde agarose gel electrophoresis was performed (Sambrook *et al.*, 1989). Sample loading buffer (15 µl, Addendum F) was added to 0,5 µg of each RNA sample, incubated at 65°C for 10 min to denature secondary structures and cooled on ice. The samples were analyzed by gel electrophoresis on 1% formaldehyde agarose gels in RNA electrophoresis buffer (Addendum F) at 65 V for approximately 1.5 h (Sambrook *et al.,* 1989).

2.8 cDNA synthesis

Total RNA was reverse-transcribed using the Transcriptor First Strand cDNA Kit (Roche Applied Science, South Africa) according to the manufacturer's protocol for cDNA synthesis using anchored oligo (dT) priming. Each RNA sample (≈1 µg) was mixed with 0.5 µl oligo(dT) pimers (final concentration 2,5 µM) and DEPC-treated water to make up a final volume of 6.5 µl. The mixture was incubated for 10 min at 65°C. Samples were put on ice and allowed to cool down. Subsequently, 2 µl Transcriptor Reverse Transcriptase Reaction Buffer, 0.25 µl Protector RNase Inhibitor (10 units), 1 µl dNTP mix (final concentration 1 mM of each dNTP)

and 0,25 µl Transcriptor Reverse Transcriptase (5 units) were added to each sample, mixed carefully and incubated at 50°C for 1 hour. To stop the Transcriptor Reverse Transcriptase samples were incubated for 5 min at 85°C. Thereafter samples were used for PCR or stored at -80°C.

2.9 Conventional PCR

Conventional PCR was performed using GoTaq buffer and GoTaq Flexi DNA polymerase (Promega Corp., USA). Each reaction contained the following:

5x GoTaq Buffer	1x
10 mM dNTP's	0.2 mM of each dNTP final
Sense and anti-sense primers	See Table 2.1
25 mM $MgCl_2$	1.5 mM
5 U/µl Go Taq Flexi Polymerase	1.25 U
Input/Template DNA	1 µl
PCR H_2O	Make up to final volume of 25 µl

The PCR protocol was as follows: Initial denaturing 95°C for 2 mins followed by 35 cycles of denaturing 95°C for 30 sec, annealing for 45 sec, extending 72°C for 45 sec, followed by a final extension 72°C for 5 min. Table 2.1 shows the gene-specific primers used for conventional PCR. Refer to Addendum B for Primer design.

Table 2.1: Sequences, concentration, annealing temperatures and product sizes of gene-specific primers used in conventional PCR.

Primer	Primer Sequence 5'-3'	Stock Concentration	Final Concentration	Annealing Temperature	Product Size
PR(A+B) forward	GGTGGGCCTTCCTAACGAG	10 µM	0.3 µM	60°C	121 bp
PR(A+B) reverse	GACCACATCAGGCTCAATGCT				
PR-B forward	GGTCCCCCTTGCTTGCA	10 µM	0.3 µM	60°C	121 bp
PR-B	CAGGACCGAGGAAAAAGCAG				

reverse					
ERα forward	GTCTGGTCCTGCGAAGGCTGCAA	10 µM	0.3 µM	60°C	235 bp
ERα reverse	GCCTTCCAAGTCATCTCTCTGACG				
GRα forward	TGCTATGCTTTGCTCCTGATCTG	10 µM	0.3 µM	52°C	299 bp
GRα reverse	TGTCAGTTGATAAAACCGCTGCC				
AR forward	GAGAACCCATTGGACTACG	10 µM	0.3 µM	52°C	544 bp
AR reverse	TGAAGAAGACCTTGCAGC				
mPRα forward	CGTTTCGGTCCACTGATCCCGG	10 µM	0.5 µM	56°C	214 bp
mPRα reverse	GCGAGAAGACCTTCGGCATGTAGATACG				

After conventional PCR, the amplified DNA was analysed on a 1.5% agarose gel by means of gel electrophoresis and visualised with ethidium bromide.

2.10 Quantitative real-time PCR

It must be noted that all gene-specific primer pairs used in conventional and quantitative real-time PCR experiments were chosen with intron spanning properties; with the exception of the PR-B and and-(A+B) primer pairs (Addendum B). This was determined using the NCBI database BLAST searches (data not shown). Intron-spanning primers were used to control for any genomic contamination that may have carried through during the preparation of RNA samples.

The authors' reproducibility in the use of quantitative real time PCR was first assessed as described in Addendum C.

Quantitative real-time PCR was performed using SensiMix^{TM} dT kit (Quantace, UK) and the Corbett real-time PCR machine and reaction tubes according to the manufacture's protocols (Quantace, UK). The reaction mixture was prepared as follows:

Sensi-MixdT	12.5 µl
50x SYBR®Green I solution	0.5 µl
Sense and anti-sense primers	(see table 2.2 for final concentration of primers)
cDNA Template	1 µl
PCR-grade H2O	Make up a total volume of 25 µl.

The PCR protocol was as follows: 95°C for 10 min followed by 40 cycles of 95°C denaturation for 10 sec, (see Table 2.2 for annealing temperatures) annealing for 10 sec, 72°C extension for 10 sec and a final 72°C for 7 min (refer to Addendum B for primer design).

Melting curve analysis and gel electrophoresis was performed to confirm the generated product in each sample. Relative gene transcript levels were calculated using the Pfaffl equation below, normalised to GAPDH transcript levels.

$$\text{Relative expression} = \frac{[E_T]^{\Delta Ct\ (control-sample)}}{[E_R]^{\Delta Ct\ (control-sample)}}$$

Where $[E_T]$ is the primer efficiency of target gene and $[E_R]$ is the primer efficiency of reference gene.

Table 2.2: Sequences, concentrations, annealing temperatures and product sizes for gene-primers used in quantitative real-time PCR.

Primer	Primer Sequence 5'-3'	Stock Concentration	Final Concentration	Annealing Temperature	Product Size
GnRHR forward	CCACAGTGGTGGCATCAGGCCTTC	5 µM	0.125 µM	58°C	192 bp
GnRHR reverse	TAGCGTTCTCAGCCGAGCTCTTGG				
ERα forward	GTCTGGTCCTGCGAAGGCTGCAA	10 µM	0.3 µM	60°C	235 bp
ERα reverse	GCCTTCCAAGTCATCTCTCTGACG				
PR-B forward	GGTCCCCCTTGCTTGCA	10 µM	0.3 µM	60°C	121 bp
PR-B reverse	CAGGACCGAGGAAAAAGCAG				
GRα forward	TGCTATGCTTTGCTCCTGATCTG	10 µM	0.3 µM	52°C	299 bp
GRα	TGTCAGTTGATAAAACCGCTGCC				

reverse					
GAPDH forward	TTCACCACCATGGAGAAGGC	5 µM	0.25 µM	58°C	263 bp
GAPDH reverse	GGCATGGACTGTGGTCATCA				

Refer to Addendum B for primer design.

Table 2.3: Primer Efficiency for primer pairs used in real-time PCR.

Primer pair	Primer Efficiency
ER	113% (E=2)
PR	75% (E=1.75)
GR	130% (E=2)
GnRHR	95% (E=1.95)
GAPDH	95% (E=1.95)

Primer efficiency was calculated for the ER, PR and GR primer pairs; (Addendum C). Primer Efficiency for GAPDH and GnRHR primers were previously determined (Kotitchke, 2009)

2.11 Western blot analysis

For over-expression (positive controls), COS-7 cells were plated in 6-well culture plates (Greiner Bio-one, International, Austria) at a density of 2 X 10^5 cells per well in 2 ml DMEM supplemented with 10% FCS and antibiotics as describedin Section 2.4. Twenty for hours after plating, medium was replaced with fresh media and cells were transfected with 1 µg pCMV-HA-hGR, pMT-PR-B and pSG5-hERα expression vectors. All transfections in the COS-7 cell line were performed using the DEAE Dextran transfection protocol adapted from Ausubel, (1999). Briefly, transfection media was prepared (2.5% FCS DMEM containing 0.1 mM chloroquinediphosphate, 1 µg/ml expression construct and 0.1 mg/ml DEAE Dextran) and 500 µl was added to each well and incubated for 1 hour at 37°C. Transfection medium was aspired and replaced with 1 ml warmed (37°) 10% (v/v) DMSO/PBS and incubated for 3 min. Thereafter the cells were gently washed with PBS, and full DMEM was replaced.

LβT2 cells were plated in 12-well culture plates (Greiner Bio-one, International, Austria) at a density of 4,5 X 10^5 cells per well in 1 ml DMEM medium supplemented with 10% FCS and

antibiotics (as describedin Section 2.4). Twenty-four hours after plating, medium was replaced with Phenol red-free Dulbecco's Modified Eagles Medium (D1145) (Sigma-Aldrich, South Africa) supplemented with 10% charcoal stripped serum and antibiotics (as described in Section 2.4). If indicated, a concentration of 0.2 nM E2 was added to each sample as shown in the appropriate figure legends. Forty-eight hours after E2 priming, the medium was replaced with phenol red free Dulbecco's Modified Eagles Medium (D1145) (Sigma-Aldrich, South Africa), serum-free media, containing antibiotics (as described in Section 2.4) and 100 nM test compounds for 8 hours, as indicated in the figure legends. Thereafter the cells were washed twice with cold PBS and harvested in 50 µl 1x SDS sample buffer buffer [5x SDS sample buffer: 100 mM Tris-HCL pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% ß-mercaptoethanol and 0.1% (w/v) bromophenolblue] (adapted from Karteris *et al.,* 2006) and incubated at 95°C for 10 min. Protein samples were separated by SDS-PAGE at 120 V in 25 mM Tris-HCL, 250 mM glycine and 0,1 % SDS, pH 8,4 (Sambrook *et al.*, 1989) using a BioRad Mini Protean^{R} II electrophoresis cell. Proteins were electro-blotted onto Hybond^{TM} ECL^{TM}(AEC Amersham Biosciences, South Africa) nitrocellulose membrane for 1 h at 180 mA (Sambrook *et al.*, 1989) using a BioRad Mini Trans-Blot^{TM} cell in transfer buffer [25 mM Tris, 200 mM glycine, 10% (v/v) methanol]. Membranes were blocked in 5% blocking solution (5% (w/v) milk powder) in Tris buffered saline (50 mM Tris, 150 mM NaCl) (TBS) containing 0.1% (v/v) Tween (TBS-Tween) for 1 h at RT and subsequently incubated with primary antibody (see Table 2.4 for specific dilutions) in blocking solution at 4°C overnight. The following day the membranes were incubated with secondary HRP conjugate antibodies (see Table 2.4 for specific dilutions) for 1 h at RT in 5% milk powder (w/v) in TBS-Tween. After antibody incubation, the membranes were washed for 15 min and 2x 5 min in TBS-Tween at RT. Thereafter the blots were kept in TBS. For consecutive detection steps, the membranes were stripped in stripping buffer [100 mM ß-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-Cl, pH 6.7] for 30 min at 65°C (Sambrook *et al.,* 1989), washed 2x 10 min with TBS-Tween and blocked for 1 h in 5% blocking solution before repeating antibody incubations as described above in Section 2.11.

Table 2.4: Dilutions of primary and secondary used in Western blot analysis

Antibody	Dilution	Secondary	Dilution
Erα (polycolonal)	1:1000 (4°C-O/N)	Rabbit	1:10000 (RT-1h)
PR-B (polycolonal)	1:1000 (4°C-O/N)	Rabbit	1:10000 (RT-1h)
hPR-B (monoclonal)	1:500 (4°C-O/N)	Mouse	1:5000 (RT-1h)
hPR (A+B) (monoclonal)	1:000 (4°C-O/N)	Mouse	1:5000 (RT-1h)
GRα (polycolonal)	1:4000 (4°C-O/N)	Rabbit	1:10000 (RT-1h)
mPRα (monoclonal)	1:2000 (4°C-O/N)	Goat	1:7500 (RT-1h)
ERK1/2 (polycolonal)	1:1000 (4°C-O/N)	Rabbit	1:10000 (RT-1h)
Flotilin-1 (polycolonal)	1:4000 (4°C-O/N)	Mouse	1:5000 (RT-1h)
HA (polycolonal)	1:1000 (4°C-O/N)	Rabbit	1:10000 (RT-1h)
B-Tubilin	1:1000 (4°C-O/N)	Mouse	1:5000 (RT-1h)

2.12 Immuno-blotting and quantification of Westerns

Immuno-blotting and ECL visualization were performed with Amersham Hyperfilm^{TM} MP high performance autoradiography film. Autoradiograms were photographed using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290. Autoradiograms signal intensities were quantified using AlphaEaseFC^{TM} Software (AlphaInnotech, USA).

2.13 mPRα protein extraction optimization

2.13.1 HEPES lysis protocol

The HEPES lysis protocol was adapted from Krietsch, (2006).

Endo-cervical cells were plated in 6-well culture plates (Greiner Bio-one, International, Austria) at a density of 2 X 10^5 cells per well in KSF culture medium supplemented with 10% bovine pituitary extract and antibiotics, as described in Section 2.4. LβT2 cells were plated on 6-well culture plates (Greiner Bio-one, International, Austria) at a density of 5 X 10^5 cells per well in DMEM culture medium supplemented with 10% FCS and antibiotics, as described in Section 2.4. At 75% confluency, the cells were washed twice with PBS, thereafter the cells were scraped and harvested with 100 µl of HEPES lysis buffer [25 µM HEPES, 5 µM EDTA, 1

µM EGTA, 50 µl NaCl, 5% (w/v) glycerol, 1% (w/v) Nonidet P-40, 1 tablet Complete Mini Protease Inhibitor Cocktail per 10 ml (Roche Applied Science, South Africa)]. Appropriate volume of 5x SDS Sample application buffer (a modification of the methods of Karteris *et al.,* 2006) [5x SDS sample buffer: 100 mM Tris-HCL pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% ß-mercaptoethanol and 0.1% (w/v) bromophenolblue] was added for a final 1x concentration. Protein samples were analysed using western blot techniques as described above.

2.13.2 CREBS lysis protocol

End-1 cells were plated in 6-well culture plates (Greiner Bio-one, International, Austria) at a density of 2 X 10^5 cells per well in KSF culture medium supplemented with 10% bovine pituitary extract and antibiotics, as described elsewhere. LβT2 cells were plated on 6-well culture plates (Greiner Bio-one, International, Austria) at a density of 5 X 10^5 cells per well in DMEM culture medium supplemented with 10% FCS and antibiotics as described elsewhere. At 75% confluency, the cells were washed twice with PBS, scraped and harvested in 100 µl of CREBS Lysis buffer [20 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 0.1% (w/v) Sodium Pyrophosphate, 1 µM β-glycerolphosphate, 1 tablet Complete Mini Protease Inhibitor Cocktail per 10 ml (Roche Applied Science, South Africa)]. An appropriate volume of 5x SDS sample application buffer [using a modification f the methods of Karteris *et al.,* (2006)], [5x SDS sample buffer: 100 Mm Tris-HCL Ph 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% ß-mercaptoethanol and 0.1% (w/v) bromophenolblue] was added for a final 1x concentration, with protein samples analysed using Western blotting techniques as described above.

2.14 Statistical analysis

Results were statistically analysed and plotted with GraphPad PRISM™ (version 5) software from GraphPad Software Inc. All real-time and quantified protein expression data was statistically analysed using non-parametric two-way ANOVAs, with a Bonferroni post-test (comparing all values to control column). Reporter assays used to investigate basal SR-transactivation (Figures 3.3, 4.3 and 5.3) were statistically analyzed using two tailed t-tests. Reporter assays used to investigate hormone GnRH and E2 priming (Figures 3.5 and 4.5)

were statistically analysed using non-parametric two-way ANOVAs, with Dunnette post-test. Where statistical significance was obtained relative to a single control, statistical significance is denoted by *, ** or ***, to indicate $P<0.05$, $P<0.01$ or $P<0.001$, respectively. All statistical calculations are represented in Addendum E

Fractional occupancy of the GR for progesterone was calculated as [ligand]/([ligand] + Ki_{P4}).

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CHAPTER 3

RESULTS AND DISCUSSION

3. The effects of Dex, P4, E2 and GnRH regulating GnRHR mRNA expression in LβT2 cells

3.1. Synergistic effects of Dex and GnRH on GRE-mediated transactivation

Glucocorticoids have been shown to increase GnRHR mRNA expression, with the ligand-dependent activation of the GR inducing endogenous GnRHR mRNA expression in LβT2 cells (von Boetticher, 2008; Kotitschke *et al.,* 2009). In addition to GCs, GnRH has also been shown to increase GnRHR expression. Previous studies from the Hapgood lab have shown that continuous treatment with 100 nM GnRH not only increases the expression of a transiently transfected mouse GnRHR reporter-promoter construct by 2.5 fold (Sadie, 2006), but also up-regulates endogenous LβT2 GnRHR mRNA expression (Kotitschke *et al.,* 2009; Sadie, 2006). In the physiological context of GnRH secretion, studies performed by Bedecarrats *et al.,* (2003) have shown that pulsatile stimulation of GnRH (1 pulse every 30 min) for 10 hours resulted in a 2-fold increase in GnRHR promoter activity and a 2-fold increase in GnRHR numbers on the cell surface of LβT2 cells (Bedecarrats and Kaiser, 2003).

It was recently shown that combined treatment with both Dex and GnRH regulate GnRHR gene expression in LβT2 cells through a mechanism involving GnRHR and GR-mediated signalling (Kotitschke *et al.,* 2009). Dex treatment alone resulted in the ligand-mediated activation of the GR, and GnRH treatment alone resulted in the ligand-independent activation of the GR and the activation of AP-1 transcription factors c-Jun and c-Fos. The study further showed that combined treatment withDex and GnRH acted synergistically to regulate GnRHR expression through mechanisms dependent on both the GR and the GnRHR. These effects were repeated on a TAT-GRE-luc reporter. Differences in the level of response

to GnRH treatments were seen on a TAT-GRE-luc reporter plasmid, however, the mechanism of transactivation on a TAT-GRE differs from that of the endogenous promoter region of the GnRHR gene. The GnRHR promoter does not contain any full GREs, but is regulated by a number of different *cis*-elements, including an AP-1 site (Figure 1.8) (Kotitschke *et al.,* 2009).

In addition to Dex and GnRH, P4 treatment has also been shown to regulate LHβ and FSHβ mRNA expression in LβT2 cells (Thackray *et al.,* 2009; An *et al.,* 2009). The suppressive action of P4 on LHβ mRNA expression is mediated by the recruitment of liganded PR to the endogenous LHβ promoter region in LβT2 cells (Thackray *et al.,* 2009). It was further shown that LHβ suppression does not require direct binding of the PR to the promoter, and thus PR is likely to be recruited to the promoter via interaction with other transcription factors (Thackray *et al.,* 2009). It was further established that PR and GnRHR crosstalk signalling up-regulates PRE-luc reporter expression in αT1-3 cells (An *et al.,* 2006) and FSHβ mRNA expression in LβT2 cells (An *et al.,* 2009). In both cases, GnRH treatment increased mRNA expression of target genes via the ligand-independent activation of the PR (An *et al.,* 2006; Thackray *et al.,* 2006). An *et al.,* (2009) showed direct binding of the PR to a PRE in the FSHβ promoter, mediated by an increase in steroid receptor co-activator NCOA3 interaction in response to GnRH in LβT2 cells (Thackray *et al.,* 2006), and in αT3-1 cells (An *et al.,* 2006). Therefore P4 treatments were included into this study to assess the potential for PR and GnRHR crosstalk in mediating GnRHR mRNA expression.

Before investigating novel crosstalk pathways, the effects of Dex and GnRH on GR-mediated GRE-luc reporter plasmid transactivation were first confirmed in the LβT2 cell line. LβT2 cells transiently transfected with a TAT-GRE-luc reporter-promoter plasmid, were treated with saturating concentrations (100 nM) of Dex, P4, GnRH and combinations thereof, for 8 hours. Thereafter a reporter-promoter assay was performed.

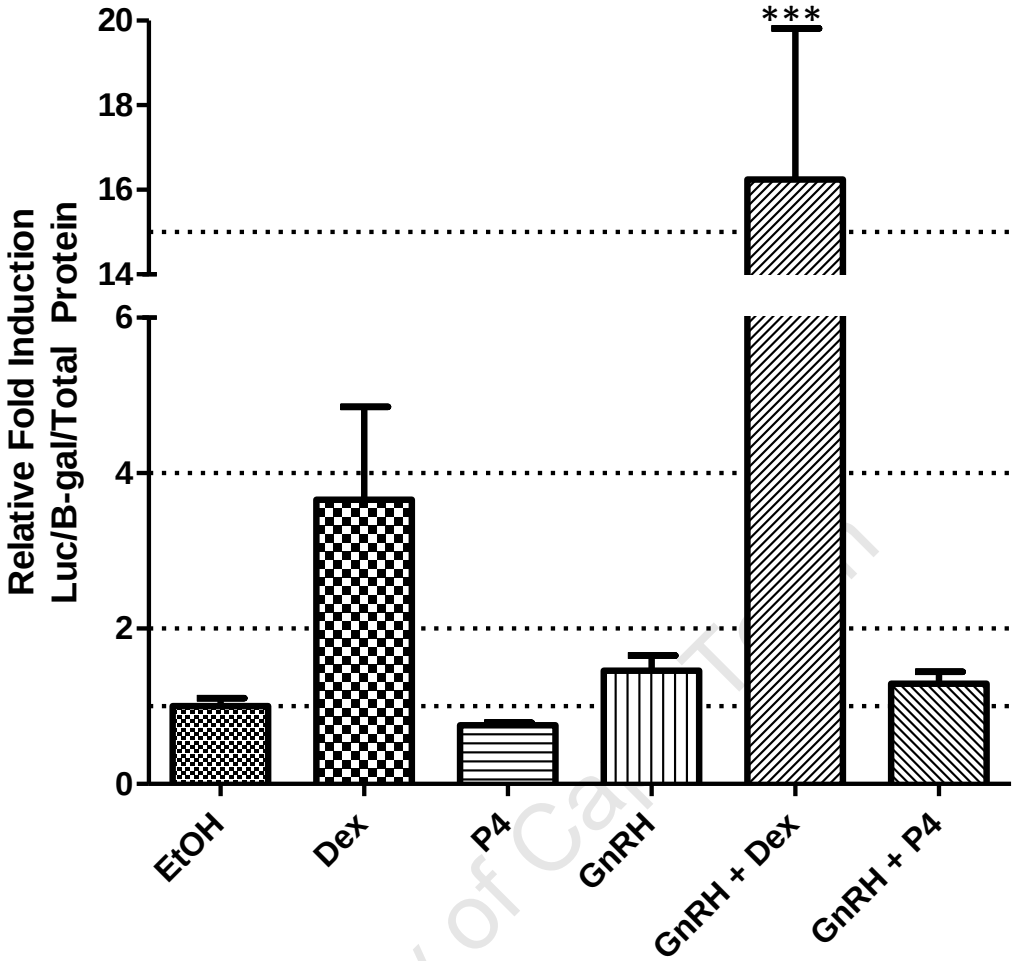


Figure 3.1: Dex and GnRH, but not P4, treatments synergistically up-regulate TAT-GRE-luc expression. LβT2 cells were seeded into a 24-well plate in DMEM supplemented with 10% serum. After a 24 hour incubation period, the medium was replaced with phenol red-free media, supplemented with 10% charcoal stripped serum and incubated for 24 hours. Thereafter the cells were transiently tranfected with 250 ng GRE-Luc reporter plasmid and 25 ng β-galactosidase expression vectors and incubated for 48 hours. After the incubation period, the medium was replaced with phenol red-free, serum-free media and the cells were the stimulated for 8 hours with vehicle (EtOH) or 100 nM of the various hormones and combinations thereof, as indicated in the figure. The cells were harvested, and luciferase and β-galactosidase assays, as well as a Bradford assay were performed. The data were normalised for transfection efficiency and cell number by expressing luciferase (luc) activity relative to β-galactosidase activity and total protein. The results represent the average of three independent experiments, performed in triplicate and presented as fold induction relative to vehicle (EtOH) control. Stars represent a significant ($P < 0.001$ = ***)

difference when compared to vehicle control, defined by a non-parametric one-way ANOVA, with a Dunnette post test.

The result shows that GR agonist Dex increases GRE-mediated transcription by about 4 fold relative to control (Figure 3.1). Although no statistical significance was observed, this is still consistent with the role of Dex, activating the GR in a ligand-dependent manner to drive GRE-promoter expression (Kotitschke *et al.,* 2009; Sadie, 2006; von Boetticher, 2008). P4 exhibits no transcriptional effects on GRE-mediated transcription, while GnRH treatment appears to increase transcriptional activity by 1.5 fold when compared to vehicle. Upon Dex and GnRH co-incubation, a significant and synergistic increase of roughly 16 fold, relative to control, is seen on GRE-mediated transcription, while P4 and GnRH appeared to have no effect on the transcriptional activity on the GRE-reporter promoter plasmid. The result of Dex plus GnRH is consistent with a previous study showing crosstalk between GR and GnRHR signalling pathways (Kotitschke *et al.,* 2009). However the lack of a significant response from GnRH treatment alone does not coincide with the finding of Kotitschke *et al.,* (2009), as it was previously reported that GnRH is able to significantly induce GRE-mediated transcription. Nevertheless the 16 fold increase seen with Dex and GnRH co-treatment (Figure 3.1) indicates that the current LβT2 cells are responding to Dex plus GnRH according to the literature, and that novel signalling pathways can be further investigated.

From Figure 3.1 it appears that 100 nM P4 treatments do not activate the GR (Figure 3.1). However, it is known that P4 is not a PR specific agonist, and has been shown to be a partial agonist for the GR (Ronacher *et al.,* 2009). Considering the relative binding affinities (RBA) of both P4 and Dex for the GR (with a Kd of 270 nM and 14 nM, respectively) (Ronacher *et al.,* 2009), concentrations of 100 nM of P4 were used to ensure PR-specific activation. The 100 nM P4 treatments should not saturate the GR, and all results with 100 nM P4 treatments should represent PR-mediated signalling and not GR-mediated signalling. Previous reports have shown that P4 regulates LHβ mRNA expression through mechanisms dependent on the PR directly binding to a PRE in the LHβ promoter in the LβT2 cell line (Thackray *et al.,* 2009). In addition, crosstalk signalling between the PR and GnRHR has been shown to regulate endogenous FSHβ gene expression in LβT2 cells (An *et al.,* 2009) and PRE-luc expression in

αT3-1 cells (An *et al.,* 2006), via GnRHR mediating the ligand-independent activation of the PR. No studies in the literature have shown the combined effects of P4 plus GnRH co-treatment on GRE/PRE-mediated transcription, or on GnRHR mRNA expression. It is known that no consensus PRE/GRE is present on the GnRHR promoter (Kotitschke *et al.,* 2009). Additional *cis*-elements in the GnRHR promoter may mediate novel effects of P4 and GnRH on GnRHR mRNA expression. Therefore the effects of P4, GnRH and combinations thereof were tested on endogenous GnRHR gene expression.

3.2 Dex and GnRH regulate endogenous GnRHR mRNA expression in LβT2 cells

Having shown that Dex plus GnRH synergistically activate transcription of a GRE-reporter promoter, the synergistic effect of Dex plus GnRH on endogenous GnRHR mRNA expression was next investigated in LβT2 cells, to confirm previous results from the Hapgood laboratory (Kotitschke *et al.,* 2009).

SR and GnRHR crosstalk mechanisms have been shown to regulate LβT2 responses (An, *et al* 2009; Chen *et al.,* 2009). For example, a PR and GnRHR crosstalk mechanism mediated by GnRH treatment, results in the rapid phosphorylation of the PR. This induces PR co-factor recruitment with NCOA3 to mediate PR binding to the Fshβ promoter, in turn up-regulating endogenous expression of the FSHβ mRNA in LβT2 cells (An, *et al* 2009). Another crosstalk mechanism between the ER and GnRHR shows the rapid phosphorylation of the ERα in response GnRH, mediates the ER to associate with the co-factor p300/CREB association factor (PCAF), in turn increasing the transcriptional activity on the endogenous fosB gene in LβT2 cells (Chen *et al.,* 2009).

In order to determine whether E2 or P4, alone or in combination with GnRH, regulate GnRHR mRNA expression, as well as to confirm previous results by others for Dex and GnRH, experiments were designed to assess the effect of the test compounds on endogenous LβT2 GnRHR mRNA expression. Previous studies in the literature have used 0.2 nM E2 priming to assess effects of E2 on PR-mediated transcription in the LβT2 cell line (Turgeon and Waring, 2006; An *et al.,* 2009). Therefore keeping in line with the current literature, LβT2 cells were primed for 48 hours with or without 0.2 nM E2 to assess the effects of E2 priming on LβT2

responses. Thereafter the cells were treated for 8 hours with vehicle or test compounds, Dex, P4, GnRH and combinations thereof, at saturating concentrations (100 nM). After ligand incubation, samples were harvested and RNA was extracted (Figure 9.6), followed by subsequent cDNA conversion. Quantitative real-time PCR with GnRHR-specific primers was performed to assess the transcriptional effects of hormones in regulating endogenous GnRHR mRNA levels in the LβT2 cell line.

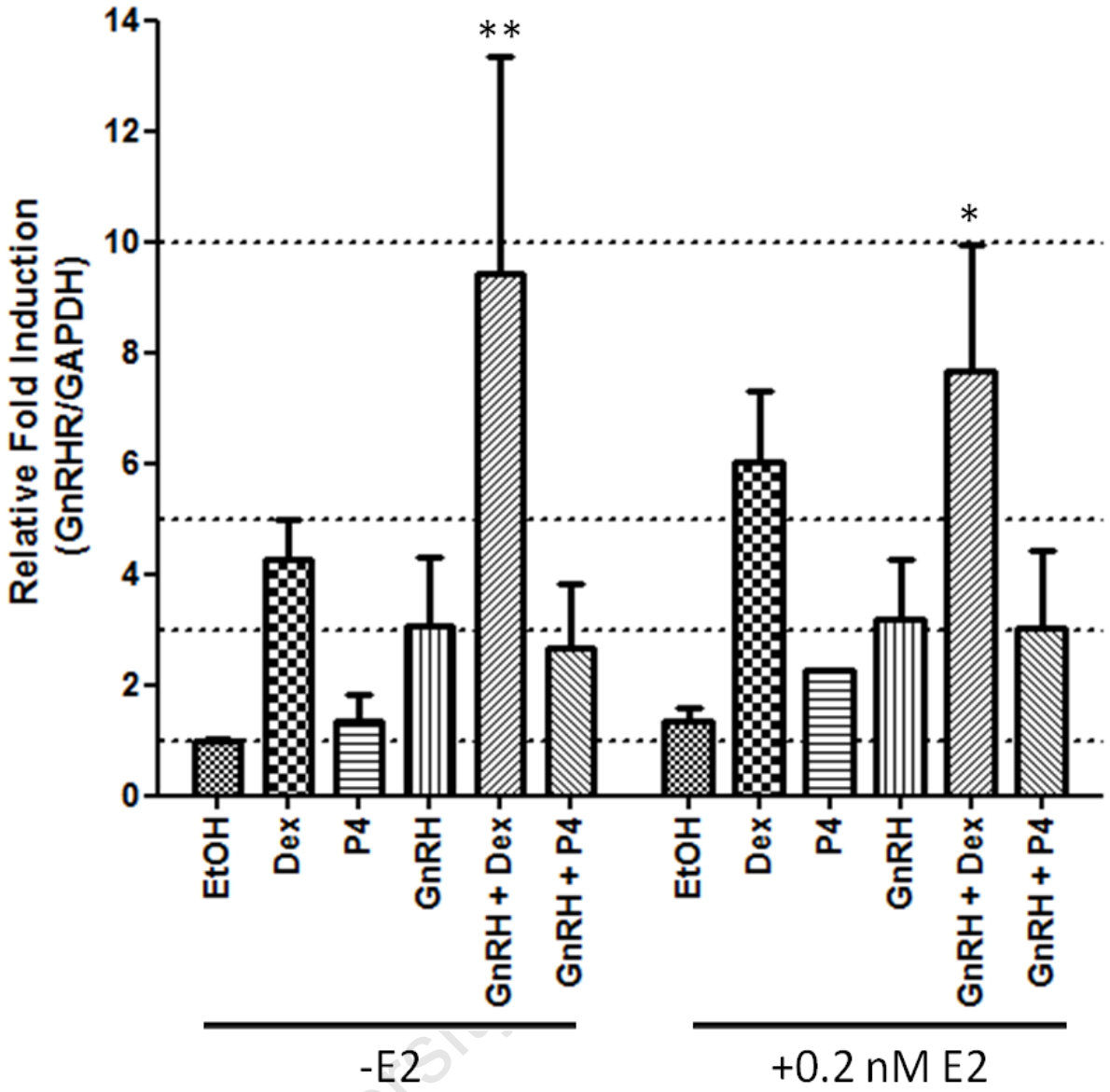


Figure 3.2: Endogenous GnRHR expression is regulated in a ligand-dependent manner. LβT2 cells were plated into 12-well plates in DMEM supplemented with 10% serum. After a 24 hour incubation period, the cells were treated with or without 0.2 nM E2 in phenol red-free medium supplemented with 10% charcoal stripped serum. After 48 hours, the cells were treated with 100 nM vehicle (EtOH) or the appropriate ligands and combinations thereof, in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting and subsequent RNA extractions. cDNA was generated for all mRNA sample sets, and was further analysed using GnRHR-specific primers and quantitative real-time PCR (Rotogene machine; Rotogene-6000). Endogenous GnRHR expression (Figure 11.5.1) was normalised to endogenous GAPDH expression (Figure 11.6.1), and is represented as fold induction relative to vehicle (EtOH). The graph shows pooled results for several independent experiments (n=4 for all samples without E2, n=3 for all E2 containing samples, and n=1 for +E2 P4 sample). Stars represent a significant ($P < 0.01$ = **) difference when compared to untreated control (-E2 EtOH), defined by a non-parametric, two-way ANOVA with a Bonferroni post test.

From Figure 3.2 it appears that endogenous GnRHR expression is regulated in a ligand-selective manner. In the absence of E2 priming, stimulation with Dex appears to result in a 4 fold increase in relative GnRHR expression, while stimulation with GnRH shows a 3 fold increase in relative GnRHR expression. Upon Dex and GnRH co-treatment, GnRHR mRNA levels significantly increased to 9.5 fold when compared to vehicle. Although the magnitude of the Dex plus GnRH response varied between individual experiments (due to the high sensitivity of gonadotrope cells to their environment and biological variation) the same trend was always observed. After much statistically analysis, it would appear that the large and varied response observed for the Dex plus GnRH response reduces the statistical significance of other responses seen. However, the Dex plus GnRH response still appears to be greater than the sum of the individual Dex and GnRH responses. These results are consistent with previous reports in the literature which showed a synergistic effect of Dex and GnRH on LβT2 GnRHR expression (Kotitschke *et al.,* 2009).

Stimulation with P4 in the absence of E2 priming did not significantly change endogenous GnRHR expression (Figure 3.2). Although P4 plus GnRH co-treatment in the absence of E2 priming resulted in a roughly 2 fold increase in GnRHR expression, this increase may be accounted for by the action of GnRH treatment alone. The effects of P4 plus GnRH on endogenous GnRHR expression have not been shown in the literature previously. This result suggests that PR and GnRHR crosstalk is not involved in regulating endogenous GnRHR mRNA expression in LβT2 cells.

The effects of E2 priming alone had no significant effect on basal GnRHR expression, when comparing the two vehicle samples (Figure 3.2). Considering the E2 primed cells, stimulation with Dex appeared to increase relative GnRHR mRNA expression to about 6 fold, as compared to the 4 fold response in the absence of E2. However the presence of E2 appeared to have no effect on the GnRH response (Figure 3.2). Interestingly, the synergistic effect of Dex and GnRH co-treatment in the absence of E2 appeared to be lost in the presence of E2. Although the Dex plus GnRH response was still the largest across the E2 primed sample set, this approximate 8 fold increase was no longer additive or of synergistic nature. It appears that E2 priming can increase the Dex-mediated response, and this effect

does not persist when Dex is co-incubated with GnRH. This result may suggest that E2 priming is able to modulate the transcriptional activity of Dex signalling on GnRHR mRNA expression. However, the statistical significance of this result (with only three data sets being collected), should be confirmed with further repeat experiments.

Stimulation of the E2 primed cells with P4 alone appeared to increase GnRHR expression by about 2 fold relative to vehicle (Figure 3.2). However this result is only from one experimental repeat and needs to be confirmed through additional repeats. Co-stimulation with P4 and GnRH resulted in roughly a 3 fold increase relative to vehicle. However, as in the case of the un-primed cells, this increase in expression may be accounted for by the effect of GnRH treatment alone on LβT2 signalling. This result supports the previous finding in Figure 3.2 that PR and GnRHR crosstalk pathways do not affect GnRHR expression in the absence or presence of E2 priming.

Taken together, these results show the existence of GR and GnRHR crosstalk pathways regulating GnRHR expression in LβT2 cells (Kotitschke *et al.,* 2009). A novel and interesting finding suggests that E2 is able to affect the Dex response. P4 and P4 plus GnRH treatments, in the absence and presence of E2, revealed no significant changes in GnRHR expression. This result was unexpected as crosstalk mechanisms between PR/ER and GnRHR have previously been shown to regulate LβT2 gene targets including FSHβ and fosB mRNA expression in LβT2 cells (An *et al.,* 2009; Chen *et al.,* 2009). However the potential that other target genes may be affected by these treatments through receptor crosstalk mechanisms is still plausible.

In conclusion, these results are in agreement with the literature. The increase in GnRHR expression with Dex and GnRH treatments alone shown above (Figure 3.2) is in agreement with previously published results in the literature regarding endogenous LβT2 GnRHR regulation. Statistical significance could not be established in the current study, most likely due to the large number of conditions. A published mechanism explaining how GnRHR mRNA expression is transcriptionally regulated by GnRH in LβT2 cells suggests the involvement of the GR. Dex induces the ligand-dependent activation of the GR resulting in GR-mediated transcription. In the presence of GnRH, GnRHR induces the site-specific phosphorylation of the GR at Ser-234, resulting in the ligand-independent activation of GR

(Kotitschke *et al.,* 2009). Upon Dex and GnRH co-stimulation, these mechanisms act synergistically to increase the transcriptional activity on the GnRHR promoter.

On the other hand, the effect of E2 priming on GR-mediated signalling is not as well established in the literature. From Figure 3.2 it appears that E2 may be able to modulate the effect of Dex treatments on GnRHR mRNA expression. This suggests a number of mechanistic possibilities. However, with the current experimental data, a precise mechanism cannot be established. What can be said is that crosstalk must exist between the ER and GR for such response to occur. Further experiments are required to assess statistical significance, and determine the possible involvement of the ER in mediating this response.

In order to further understand the responses seen from Figure 3.2, an assessment of the overall effect these ligand treatments may have on SR expression levels would be required. Therefore the effect of Dex, P4, GnRH, E2 and combinations treatments thereof were tested on the relative expression levels of the SRs (ER, PR and GR) in LβT2 cells, to get a more detailed picture on the effects various ligand treatments have on LβT2 physiology. This rationale leads to a study of the ligand-selective effects on ER, PR and GR expression levels.

CHAPTER 4

RESULTS AND DISCUSSION

4. Regulation of ERα expression and function in LβT2 responses

As shown earlier, E2 priming treatments appear to affect the Dex response regulating GnRHR mRNA expression (Figure 3.2), indicating potential crosstalk between the ER and the GR. Although E2 had no effect on the GnRH-mediated increase of GnRHR expression in LβT2 cells (Figure 3.2), ER and GnRHR crosstalk pathways have been shown to regulate fosβ expression in LβT2 cells, through the rapid GnRH-induced phosphorylation of ERα (Chen *et al.,* 2009). In addition, PR-B was shown to recruit a repressor complex to a half-PRE site in the ERα gene promoter in MCF-7 human breast cancer cells (Amicis *et al.,* 2009). Therefore the question of whether Dex, P4, GnRH, E2 and combinations thereof affect ERα mRNA or protein expression levels in LβT2 cells was next examined.

4.1 Characterisation of endogenous ERα mRNA and protein expression in LβT2 cells

Before examining the effect of hormone treatments on ERα mRNA and protein expression, untreated LβT2 cells were first used to characterise ERα mRNA levels under basal conditions. LβT2 cells were cultivated under basal conditions, and harvested. Subsequent RNA extractions and cDNA conversions were performed. Thereafter, conventional PCR with intron spanning ERα-specific primers was used to assess basal *ERα* expression in LβT2 cells.

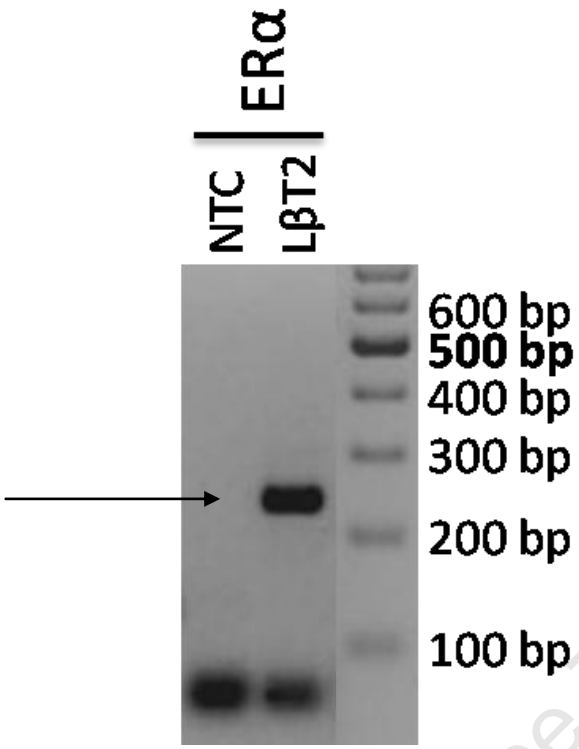


Figure 4.1: Endogenous ERα mRNA is basally expressed in LβT2 cells. LβT2 cells were seeded into 6-well plates in DMEM supplemented with 10% serum, and were left to grow to 70% confluency under basal conditions. Thereafter the cells were harvested and RNA extractions were performed, followed by a reverse transcriptase reaction to generate LβT2 cDNA. Signals were amplified using conventional PCR with ERα-specific primer pairs. PCR products were generated over 35 PCR cycles and separated on a 2% agarose gel using electrophoreses, and visualised with ethidium bromide staining. The arrow marks the position of ERα amplicon at 235bp. NTC defines the no template control.

From Figure 4.1 it is evident that *ERα* mRNA is expressed under basal conditions in LβT2 cells. This is in agreement with the current literature regarding the presence of ERα mRNA (Schreihofer *et al.,* 2000) and ER function in mediating LβT2 responses (Chen *et al.,* 2009).

Having shown the presence of ERα mRNA in LβT2s cells, it was next determined whether LβT2 cells have detectable levels of ERα protein. In order to determine this, crude cell lysates were generated from cultivated LβT2 cells, and ERα protein levels were visualised

using western blotting techniques and a specific anti-ERα antibody. COS-7 cells over-expressing hERα were harvested and investigated alongside the LβT2 lysates on the western blot, to serve as a positive control in assessing ERα protein levels in LβT2 cells.

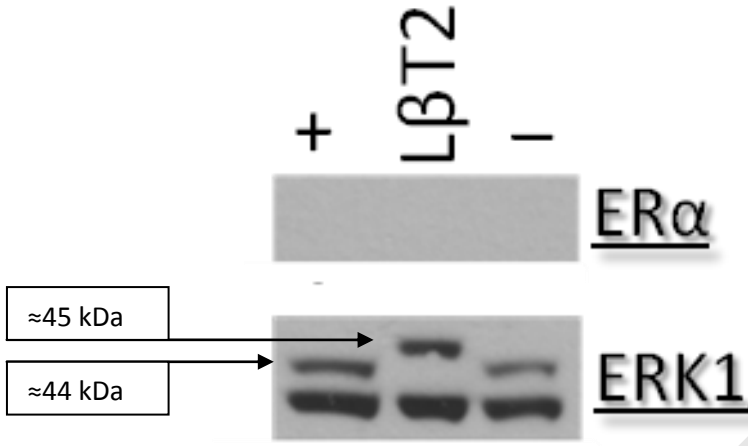


Figure 4.2: Endogenous ERα protein levels were not detected by Western blotting. COS-7 cells were seeded into 6-well plates in DMEM supplemented with 10% serum. After a 24 hour incubation, the cells were either transiently transfected with 1 µg of pSG5-hERα expression vector (positive COS-7 control) (+), or left to grow under basal conditions (negative COS-7 control) (-). LβT2 cells were seeded into a 6-well plate in DMEM supplemented with 10% serum, and left to grow under basal conditions. After all cells had reached 70% confluency (at least 24 hours after transfection), the COS-7 cells and untreated LβT2 cells were harvested in 50 µl SDS sample application buffer. Equal volumes (10 µl) of all lysates were separated on 8% SDS polyacrylamide gel, transferred to a nitrocellulose membrane and probed with anti-ERα-specific and anti-ERK1/2-specific (Loading control) primary polyclonal antibodies. Anti-rabbit-HRP secondary antibodies were used for detection of both ERα and ERK1/2 primary antibodies. The signals were visualised with Amersham Chemiluminescence. ERK1/2 proteins observed 42 kDa & 44 kDa, respectively. Arrows indicate observed difference in molecular weight between LβT2 and COS-7 ERK 2 proteins.

Although ERα protein could not be detected in LβT2 cells at the 66 kDa marker, a signal could also not be detected for the positive control (COS-7 cells over-expressing pSG5-hERα plasmid) (Figure 4.2), indicating 2 possible outcomes. The first and more likely is that the current ERα antibody used is defective and specific immuno-detection properties

compromised. The second possibility is that the ERα expression plasmid did not over-express in the COS-7 positive control and that LβT2 cells have low amounts of ERα protein that are undetectable using Western blotting. Therefore ERα protein levels could not be detected for further assessment of the response to Dex, P4, GnRH, E2 and combination treatments.

An additional observation is the difference in ERK 2 migration through the polyacrylamide gel between LβT2 and COS-7 lysates (Figure 4.2). The top band (representing ERK 2 protein) was shown to be at a slightly higher position in mice LβT2 cells (≈45 kDa), when compared to monkey COS-7 cells (≈44 kDa). This may be due to tissue-specific and species-specific differences. A similar observation was made for ERK1/2 levels, in all subsequent western blots, when characterising SR protein levels.

Having been unsuccessful in detecting ERα protein by means of western blotting, a different strategy was implemented to assess functional ERα protein expression. ERα transactivation was investigated using an ERE-luc reporter assay in the LβT2 cell line. Therefore LβT2 cells were transiently transfected with an ERE-luc reporter plasmid, and treated with ER agonist (E2) at saturating concentrations (100 nM) for 8 hours.

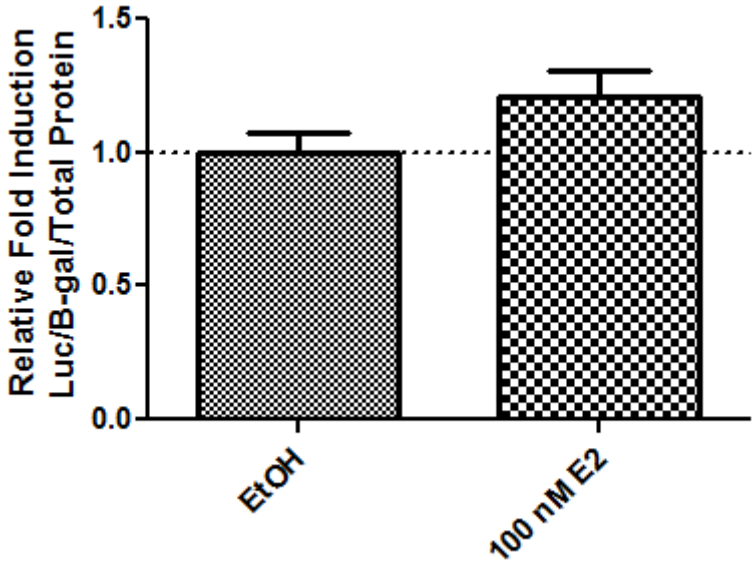


Figure 4.3: Endogenous ERα shows no functional activity on an ERE-luc in LβT2 cells. LβT2 cells were seeded into a 24-well plate in DMEM supplemented with 10% serum. After a 24 hour incubation period, the medium was replaced with phenol red-free media, supplemented with 10% charcoal stripped serum and incubated further for 24 hours. Thereafter the cells were transiently transfected with 300 ng ERE-Luc reporter plasmid and 25 ng β-galactosidase expression vector and incubated for an additional 48 hours. After the incubation period, the medium was replaced with phenol red-free, serum-free media and the cells were the stimulated for 8 hours with vehicle (EtOH) or 100 nM ER-specific agonist E2. After the cells were harvested, luciferase and β-galactosidase assays, as well as a Braford assay, were performed. The data was normalised for transfection efficiency and cell number by expressing luciferase (luc) activity relative to β-galactosidase activity and total protein. The graph shows pooled results from two independent experiments, each performed in triplicate, which is presented as fold induction relative to vehicle (EtOH) control.

Figure 4.3 illustrates that on treatment with saturating concentrations (100 nM) of ER agonist (E2), no ER-mediated transactivation was seen in the LβT2 cell line. This result suggests LβT2 cells lack functional ER protein, which is in contrast with previous findings in the literature showing the presence of functional ERα protein in LβT2 cells (Chen *et al.,* 2009; Schreihofer *et al.,* 2000). It is possible that LβT2 cells may have functional ER, however, technical complications may have occurred during the E2 stimulation or ERE-luc

transfections resulting in a false negative result. For example, transfection of the ERE-luc reporter plasmid into the LβT2 cells may have been unsucsessful.

4.2 ERα mRNA levels are regulated by GnRH in a ligand-dependent manner in LβT2 cells

While no functional ERα protein was observed by western blot and functional reporter assay (Figures 4.2 and 4.3), endogenous mRNA was detected (Figure 4.1). Technique sensitivity may have accounted for the lack of detection as western blotting and reporter promoter techniques failed to allow detection of functional ER protein. For this reason real-time PCR was used to assess the transcriptional regulation of LβT2 *ERα* mRNA levels in response to ligand treatments.

LβT2 cells were either pre-treated with 0.2 nM E2 or left untreated for 48 hours, followed by an 8 hour treatment with saturating concentrations (100 nM) of Dex, P4, GnRH and combinations thereof. Thereafter cells were harvested and RNA was extracted (Figure 9.6), followed by subsequent cDNA conversion. Quantitative real-time PCR with ERα-specific primers were performed to assess the transcriptional effects of hormones in regulating endogenous *ERα* mRNA levels in LβT2 cells.

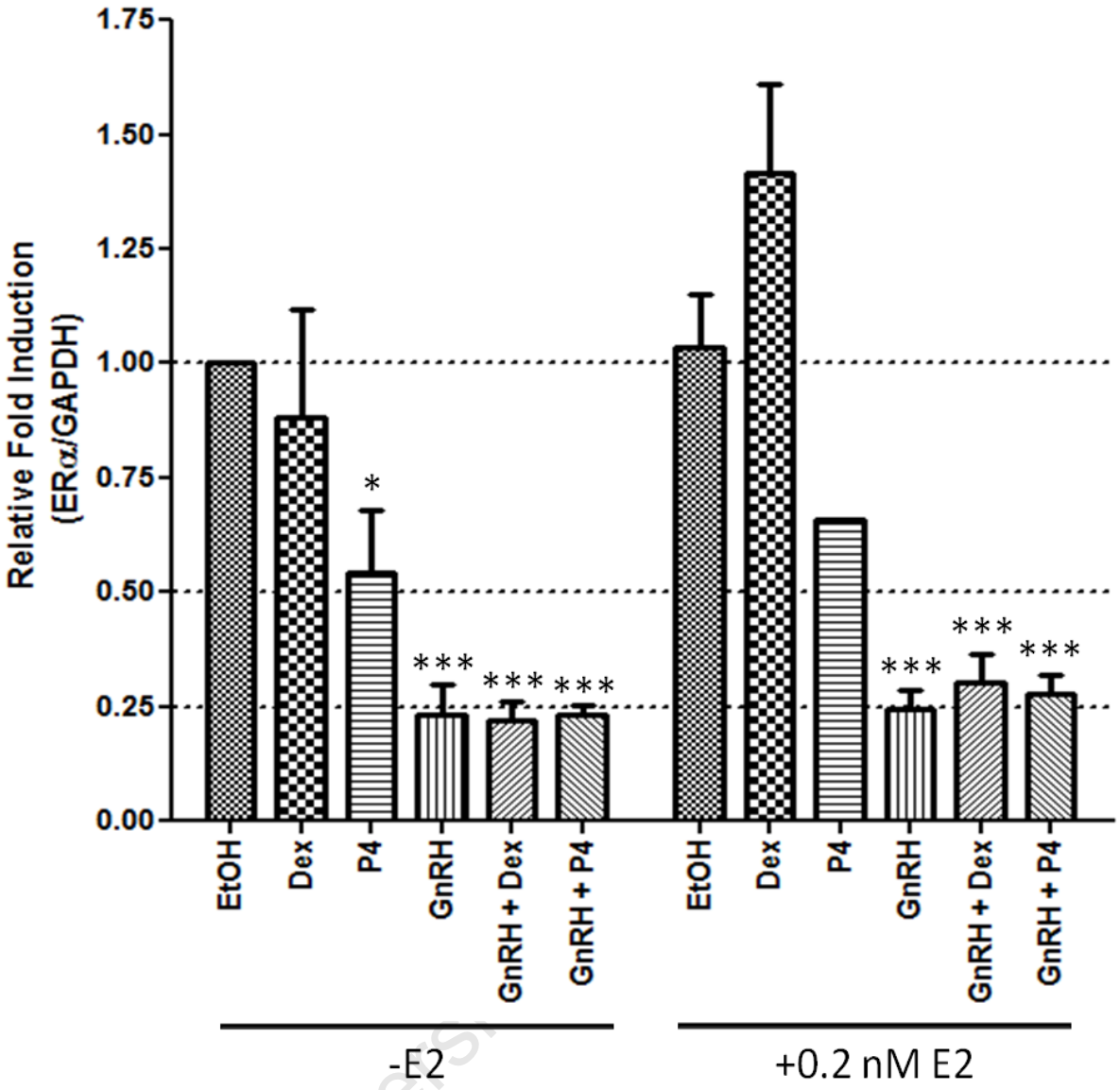


Figure 4.4: GnRH and P4, but not Dex and E2 treatment suppresses endogenous ERα mRNA expression levels in LβT2 cells. LβT2 cells were plated into 12-well plates in DMEM supplemented with 10% serum. After a 24 hours incubation period, the cells were treated with 0.2 nM E2 in phenol red-free media supplemented with 10% charcoal stripped serum or left untreated. After 48 hours, the cells were treated with 100 nM vehicle (EtOH) or the appropriate ligands or combinations thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting and subsequent RNA extractions. cDNA was generated for all mRNA sample sets and was further analysed using ERα-specific primers and quantitative real-time PCR (Rotogene machine; Rotogene-6000). Endogenous ERα mRNA expression (Figure 11.7.1) was normalised to endogenous GAPDH mRNA expression (Figure 11.6.1), and was represented as fold induction relative to vehicle (EtOH). The graph shows pooled results for several independent experiments (n=4 for all samples without E2, n=3 for all E2 containing samples, and n=1 for +E2 P4 sample). Stars represent a

significant ($P < 0.05$ = *; $P < 0.01$ = **; $P < 0.001$ = ***) difference when compared to untreated control (-E2 EtOH), defined by a non-parametric, two-way ANOVA with a Bonferroni post test.

From Figure 4.4 it is apparent that endogenous *ERα* mRNA expression is regulated in a ligand-specific manner. Dex treatments revealed no significant changes in *ERα* expression, while stimulation with P4 reduced relative *ERα* mRNA levels to 0.55 fold expression. The suppressive effect of P4 on ERα mRNA expression is consistent with the literature showing P4 has suppressive effects on ER expression and function in breast cancer cells (Graham and Clarke, 1997). However, it has not been previously investigated in LβT2 cells. Treatment with GnRH also showed a significant suppressive effect on ERα gene transcription, decreasing *ERα* mRNA levels to 0.25 fold relative to vehicle.

Co-treatments with GnRH plus Dex or with GnRH plus P4 resulted in a similar suppressive effect as observed for GnRH treatment alone, where both co-incubations resulted in *ERα* mRNA levels decreasing significantly to 0.25 fold relative to vehicle (Figure 4.4). The suppressive effects of Dex plus GnRH co-treatment can be accounted for through GnRH treatment alone, as no differences are seen between the GnRH plus Dex and GnRH treatments. Although it appears that P4 alone, and GnRH alone both significantly represses *ERα* mRNA expression, co-incubation with both P4 and GnRH show no additive repressive properties, suggesting two possibilities. One possibility is that GnRH and P4 signalling are not functionally integrated in regulating *ERα* mRNA expression. The other is that both GnRH and P4 alone maximally inhibit *ERα* mRNA expression to the extent that additional treatment no longer has a repressive effect, as the expression of *ERα* cannot be suppressed beyond maximum. Further experiments using lower concentrations of GnRH (i.e. 1 nM) during GnRH and P4 co-treatment should discriminate between the two possibilities.

It appears that E2 priming alone has no significant effect on *ERα* mRNA expression in LβT2 cells (1.1 fold), as compared to unprimed E2 vehicle (Figure 4.4). This result was unexpected since it was previous shown that E2 has a suppressive effect on ERα mRNA and protein expression in primary rat pituitary cells (Schreihofer *et al.,* 2000). However, this may be because different cells and different treatment conditions were used in these studies.

Perhaps an effect would be seen if LβT2 cells were treated with E2 at a saturating concentration of 100 nM E2 (as used by Schreihofer *et al.,* (2000). The IC_{50} for the ERα binding to E2 has been determined experimentally to be 0.9 nM (Blair *et al.,* 2000). Therefore a concentration of 0.2 nM E2 should not result in an ERα-specific response. Furthermore treatment with, E2 and P4 had no significant effect on the receptor GnRHR mRNA expression (Figure 3.2). Therefore it appears that the repressive effects of E2 and P4 in LβT2 cells are gene-specific, repressing ERα and LHβ (Schreihofer *et al.,* 2000; Thackray *et al.,* 2009), but not GnRHR levels.

Stimulation of E2 primed cells with Dex shows an apparent, although not significant, increase in *ERα* mRNA expression of 1.4 fold relative to vehicle, as compared to the un-primed Dex treatment (0.8 fold) (Figure 4.4). This is the second time that E2 has affected the outcome of a Dex response in this study, the first seen in the GnRHR mRNA expression result (Figure 3.2). This apparent change in *ERα* mRNA expression may be a result of several possibilities. One possibility suggests that E2 may increase GR levels, in turn increasing Dex responsiveness on ERα mRNA transcription. Another possibility suggests that liganded ER may directly interact with the GR, in turn increasing the transcriptional activity of the GR through the formation of a GR/ER complex on the ERα promoter (Cvoro *et al.,* 2011). A final possibility may suggest E2 (via the ER) may indirectly affect the phosphorylation status of the GR through a possible change in kinase or phosphatase activity (Zhang *et al.,* 2009). Taken together this result suggests that low dosages of E2 may influence the transcriptional outcome of Dex treatments on GR-regulated genes, suggesting potential crosstalk between the ER and the GR in LβT2 responses. Further experiments will be needed to establish whether this increase is statistically significant, and to determine the molecular mechanism governing the effect of E2 priming influencing Dex-induced GR transcriptional regulation.

The effects of P4 on E2 primed LβT2 cells still show a reduction in relative *ERα* mRNA expression (0.65 fold) when compared to the un-primed sample set (0.55 fold) (Figure 4.4). However, it should be noted that this result is from one experiment and thus additional repeat experiments are required to determine whether this response is reproducible. From the single P4 (+E2) treatment, it nevertheless appears that E2 priming has no effect on the P4-mediated suppression of *ERα* mRNA expression.

Stimulation with GnRH on +E2 primed LβT2 cells significantly reduced *ERα* mRNA levels to 0.25 fold relative to vehicle, similar to the effects of GnRH treatment on un-primed LβT2 cells (Figure 4.4). Therefore it appears that E2 priming has no effect on the GnRH-mediated down regulation of endogenous *ERα* mRNA expression in LβT2 cells. Similarly, the effects of GnRH co-treatment with Dex or P4 on E2 primed LβT2 cells show no differences when compared to the unprimed samples set (Figure 4.4). Both hormone combination treatments reduce *ERα* mRNA levels to 0.3 fold relative to vehicle, and are most likely due to the suppressive effects of GnRH treatment alone in regulating endogenous *ERα* mRNA expression in LβT2 cells. To the author's knowledge, no previous studies in the literature have shown the effects of E2 priming on Dex and GnRH signalling in LβT2 responses.

In conclusion, GnRH treatments had the most significant effect in regulating endogenous *ERα* mRNA expression in LβT2 cells. Treatment with GnRH alone resulted in a significant 25-30% reduction in relative mRNA expression levels (Figure 4.4), with E2 priming having no effect. To the author's knowledge, little is known about the precise mechanism of GnRH-induced *ERα* mRNA suppression in LβT2 cells. It is possible that a transcription factor may be phosphoryated and activated to regulate *ERα* mRNA expression in response to GnRHR-mediated signalling. Interestingly, previous studies have shown the presence of an AP-1 site (Tang *et al.,* 1997), a half-PRE/GRE site (Amicis *et al.,* 2009), and a palindomic binding site for ERF-1 (McPherson *et al.,* 1997), however, no full ERE has been found in the promoter region of the ERα gene. However, the precise mechanism of action cannot be predicted with the current data, yet this will most likely involve these *cis*-elements in the ER promoter region.

P4 also has a suppressive effect on *ERα* expression, reducing levels to about 45 % relative to vehicle in LβT2 cells. The anti-estrogenic action of P4 has been previously established in MCF-7 breast cancer cells, with liganded PR recruiting specific repressor complexes to a half-PRE/GRE site in the ERα gene promoter region (Amicis *et al.,* 2009). P4-mediated inhibition of *ERα* mRNA expression has also been shown in a number of other reproductive tissues including breast cells (Read *et al.,* 1989; Alexander *et al.,* 1990), and the uterus (Master *et al.,* 1974; Hsueh *et al.,* 1975), yet has not been shown in gonadotrope cells. Futhermore the co-incubation of GnRH plus P4 shows no additive or synergistic repressive properties,

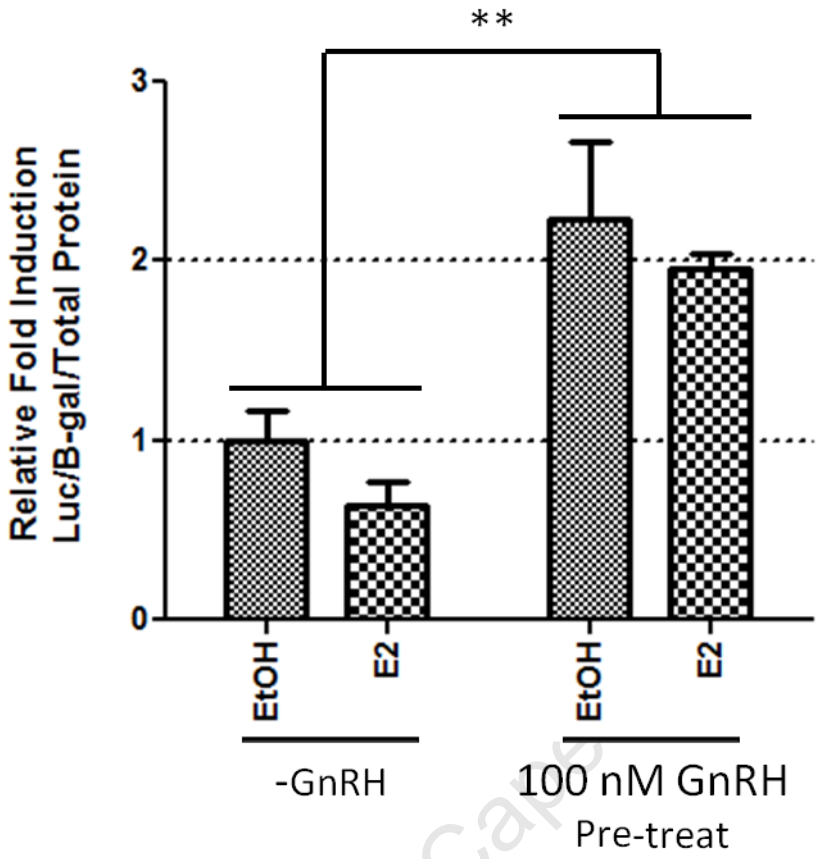
suggesting that the GnRH and P4 signalling pathways do not crosstalk in regulating *ERα* mRNA expression at the level of the pituitary.

Finally E2 priming appeared to modulate the Dex response regulating *ERα* mRNA expression. Stimulation of E2 primed LβT2 cells with Dex appeared to show an increase in *ERα* mRNA levels, compared to Dex treatment alone. Although this result is not statistically significant, it appears that low doses of E2 may influence the transcriptional outcome of GR-mediated signalling. This further supports the idea that E2 priming is able to modulate the Dex responses as seen in GnRHR mRNA expression (Figure 3.2). No studies regarding the effects of E2 and Dex co-incubations on ERα and GnRHR mRNA expression have been found in the literature, indicating a novel result that should be considered for further research.

4.3 The effects of GnRH priming on ERE-luc reporter expression

No functional ERα protein could be detected and no increase in ERE-reporter activity was seen in response to E2 in LβT2 cells (Figures 4.2 and 4.3). Therefore the question was asked, does GnRH modulate ER activity via an ERE in the absence and presence of E2.

LβT2 cells transiently transfected with ERE-luc reporter plasmid were either pre-treated with GnRH or left untreated for 24 hours prior to 8 hour stimulation with an ER-specific agonist (E2) at saturating concentrations (100 nM).



Treatment	Mean	Std. Dev.
EtOH	1.000	0.239
E2	0.637	0.239
(100nM GnRH pre-treat) EtOH	2.232	0.754
(100nM GnRH pre-treat) E2	1.962	0.128

Figure 4.5: GnRH pre-treatment increases basal transcriptional activity on an ERE-Luc in LβT2 cells. LβT2 cells were seeded into a 24-well plate in DMEM supplemented with 10% serum. After a 24 hour incubation period, the medium was replaced with phenol red-free media, supplemented with 10% charcoal stripped serum and incubated further for 24 hours. Thereafter 300 ng ERE-Luc reporter plasmid and 25 ng β-galalactosidase expression vector was transiently transfected and the cells were incubated with or without 100 nM GnRH for an additional 24 hours. After the incubation period, medium was replaced with phenol red-free, serum-free media and the cells were the stimulated for 8 hours with vehicle (EtOH) or 100 nM ER specific agonist E2. After harvesting the cells, luciferase, β-galactosidase and a Brafords assay was performed. The data was normalised for transfection efficiency and cell number by expressing luciferase (luc) activity relative to β-galactosidase activity and total protein. The graph shows pooled results from three independent experiments, each

performed in triplicate, which is presented as fold induction relative to vehicle (EtOH) control. Stars represent a significant (P < 0.01 = **) defined by a non-parametric, two-way ANOVA with a Bonferroni post test.

Considering ER agonist treatment alone, a small, but statistically insignificant, decrease in ER-mediated transactivation was observed (Figure 4.5). This result suggests a lack of ER-mediated activity in the current LβT2 cell line. It contradicts conventional models of ER activity, where E2 ligand-dependently activates the ER to drive ERE-mediated transcription (Edwards, 2005), which has been shown to regulate a number of LβT2 responses (Schreihofer *et al.,* 2000; Chen *et al.,* 2009). This result was previously discussed in Figure 4.3.

Nevertheless, the overall effect of a 24 hour GnRH pre-treatment on ERE-reporter activity revealed a significant increase in basal activity of roughly 2 fold relative to vehicle (Figure 4.5). The fact that the ERE-luc reporter is responsive to GnRH treatments indicates a functional ERE-luc reporter plasmid. This increase in basal transcriptional activity may be due to a general increase in basal transcription-factor recruitment to the ERE-luc reporter mediated by the GnRHR signalling pathway. GnRH pre-treated samples subjected to saturating concentration (100 nM) of ER specific agonist (E2) treatment show no significance difference compared to its internal control (100 nM GnRH pre-treat EtOH).

In conclusion, GnRH pre-treatment induced an increase in basal promoter activity (Figure 4.5) in the absence of E2 in these cells. Whether this response is mediated by endogenous ER, via the ERE site in the reporter gene, still need to be determined. Given the undetectable response to E2, it is possible that the cells contain a mutated version of the ER that is unable to respond to E2, but can be activated by GnRHR, to increase transcription via binding to the ERE. This mechanism is possible, since Chen *et al.,* (2009) showed that GnRH treatment mediates the site-specific phosphoylation of ERα protein to increase co-activator recruitment with the p300/CBP-associated factor (PCAF). These effects of GnRH on endogenous LβT2 fosB mRNA expression are dependent on the ER, and mediated by an ERE in the target gene promoter region (Chen *et al.,* 2009). However, it is also possible that the

response observed is independent of the ER, and may be mediated by GnRH activating component(s) of the basal transcription machinery present on the ERE-reporter construct. Future experiments using a mutated ERE within this construct should discriminate between these two possibilities.

CHAPTER 5

RESULTS AND DISCUSSION

5. Regulation of PR-B expression and function in LβT2 responses

As mentioned in Chapter 3, P4 treatment has been shown to have suppressive effects on GnRH signalling (Thackray.e*t al.* 2009), suggesting hormone treatments may influence PR and GnRHR signalling to regulate LβT2 gene expression. A recent study has shown the existence of such a PR and GnRHR crosstalk mechanism regulating Fshβ mRNA expression in LβT2 cells (An *et al.,* 2009). This mechanism involves the rapid phosphorylation of the PR in response to GnRH treatment, in turn activating unliganded PR to recruit transcriptional co-factors to modulate FSHβ expression.

Therefore crosstalk between the GR/PR/ER and the GnRHR, in mediating the regulation of LβT2 target genes, including GnRHR*,* FSHβ and fosβ mRNA expression, respectively (Kotitschke *et al.,* 2009; An *et al.,* 2009; Chen *et al.,* 2009), highlight the potential existence of such a mechanism for regulating target LβT2 PR gene expression.

Having shown that both P4 and GnRH appear to effect *ERα* mRNA expression (Figure 4.4), while GR and GnRHR crosstalk was shown to mediate GnRHR mRNA expression (Figure 3.2), the role of these signalling pathways on *PR* expression was next examined. The effects of E2 on *PR*-B mRNA expression were also assessed using 0.2 nM E2 priming. With regards to the literature, E2 priming has been shown to regulate PR mRNA expression in primary mice and rat pituitary cells (Turgeon and Waring *et al.,* 2006).

5.1 Characterisation of endogenous PR-B in LβT2 cells

Before examining the role of hormone signalling on *PR* expression, untreated LβT2 cells were used to characterise *PR*-A and *PR-B* mRNA levels under basal conditions. LβT2 cells were cultivated under basal conditions, and harvested. Subsequent RNA extractions and cDNA conversions were performed to generate basal LβT2 cDNA. Thereafter conventional PCR using PR-B-specific and PR-(A+B)-specific primers was used to assess basal *PR* expression in LβT2 cells. The PR-B primers were designed to anneal and amplify a 121 bp amplicon from the unique 5' end of the PR-B mRNA transcript, while the PR-(A+B) primers were designed to anneal and amplify a 121 bp amplicon from the shared 3' end of the PR-A and PR-B mRNA transcripts (Addendum A, Figure 9.7) (Turgeon *et al.,* 2006).

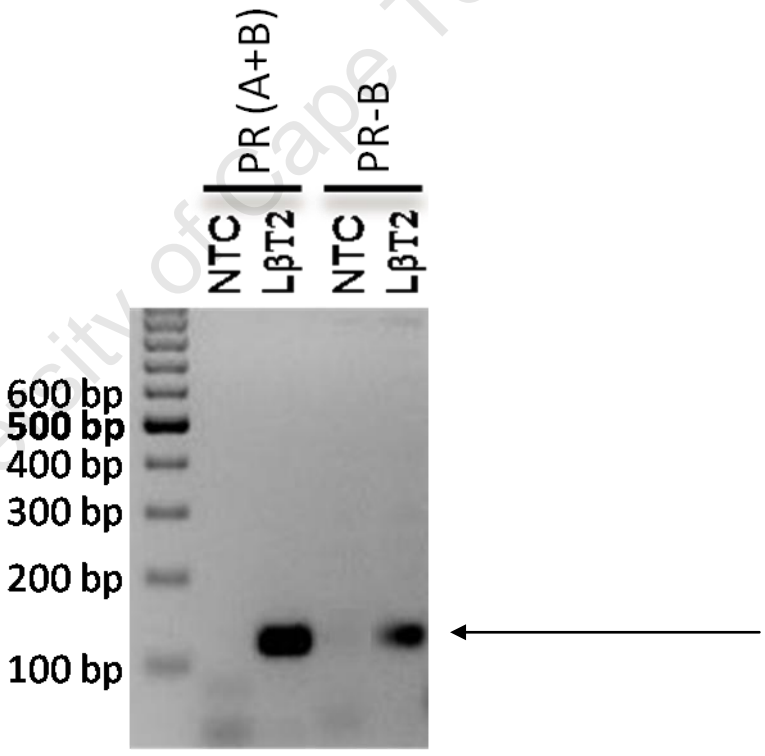


Figure 5.1: Endogenous PR mRNA is basally expressed in LβT2 cells. LβT2 cells were seeded into 6-well plates in DMEM supplemented with 10% serum, and were left to grow to 70% confluency under basal conditions. Thereafter the cells were harvested and RNA extractions were performed, followed by a reverse transcriptase reaction to generate basal LβT2 cDNA. Signals were amplified using conventional PCR with PR (A+B)- and PR-B-specific primer pairs. PCR products were generated over 35 PCR cycles, separated on a 2% agarose gel using electrophoreses and visualised with ethidium

bromide staining. The arrow indicates the presence of PR (A+B) aplicon at 121bp and PR-B aplicon at 121bp. NTC defines the no template control.

From Figure 5.1 it appears that the mRNA for both PR isoforms is expressed under basal conditions, which is in agreement with current literature regarding endogenous LβT2 *PR* mRNA expression (Turgeon and Waring, 2006). PR-B is the more "transcriptionaly active" isoform (Graham and Clarke, 1997). Therefore this study will focus on the ligand-mediated transcriptional effects on PR-B mRNA expression. Specific detection of PR-B mRNA expression is possible via PR-B-specific primers directed against the 5' end of the PR transcript, as this is absent in the PR-A isoform.

Having shown the presence of PR-B mRNA in LβT2s cells, it was next determined whether the LβT2 cells express detectable levels of PR-B protein. In order to determine this, crude cell lysates were generated from cultivated LβT2 cells, and subjected to Western blotting and probed with specific PR-B- and ERK1/2(loading control)-antibodies. Lysates from COS-7 cells over-expressing hPR-B were harvested and run alongside LβT2 lysates on the western blot to serve as a positive control in assessing PR-B protein levels in LβT2 cells.

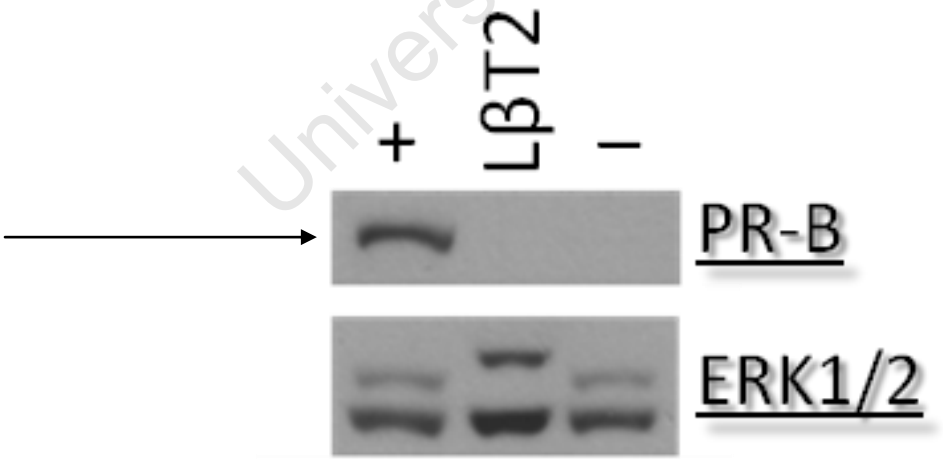


Figure 5.2: Endogenous PR-B protein is not detectable in LβT2 cells by western blotting. COS-7 cells were seeded into 6-well plates in DMEM supplemented with 10% serum. After a 24 hour incubation, the cells were either transiently transfected with 1 µg of pMT-hPR-B expression vector (positive COS-7 control) (+), or left to grow under basal conditions (negative COS-7 control) (-). LβT2 cells were

seeded into a 6-well plate in DMEM supplemented with 10% serum, and left to grow under basal conditions. After all cells had reached a 70% confluency (at least 24 hours after transfection), the COS-7 cells and untreated LβT2 cells were harvested in 50 µl SDS sample application buffer. Equal volumes (10 µl) of all lysates were separated on 8% SDS polyacrylamide gel, transferred to a nitrocellulose membrane and probed with PR-B-specific and ERK1/2-specific (Loading control) primary polyclonal antibodies. Anti-rabbit-HRP secondary antibodies were used for detection of both PR-B and ER1/2 primary antibodies. Signals were visualised with Amersham Chemiluminescence. Arrows indicate PR-B protein at 116 kDa. ERK1 and 2 proteins are seen at 42 kDa & 44 kDa, respectively.

Although PR-B protein was not detected in LβT2 cell lysates, a signal was detected for the positive control (COS-7 cell lysate over-expressing pMT-hPRβ plasmid) (Figure 5.2), suggesting that the PR-B antibody is effective in detecting PR-B protein. Because *PR*-B mRNA is detectable under basal conditions in LβT2 cells (Figure 5.1), suggests that PR-B protein is either not expressed or expressed at levels too low for detection using western blotting techniques.

Therefore, a potentially more sensitive reporter-promoter assay was used to assess functional PR protein using the PR-specific agonist, R5020. LβT2 cells were transiently transfected with a TAT-GRE-luc reporter plasmid, and treated with PR agonist (R5020) at saturating concentrations (100 nM) for 8 hours to assess PR-mediated transactivation in LβT2 cells.

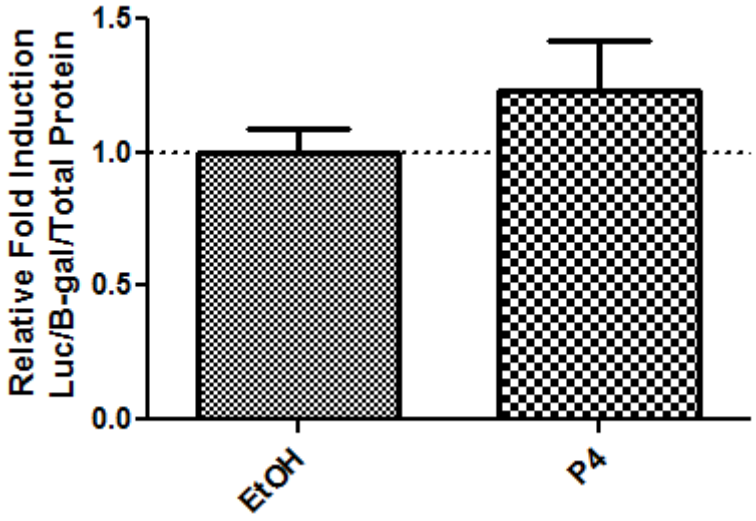


Figure 5.3: Functional reporter assay did not detect endogenous PR-mediated transactivation in LβT2 cells. LβT2 cells were seeded into a 24-well plate in DMEM supplemented with 10% serum. After a 24 hour incubation period, the medium was replaced with phenol red-free media, supplemented with 10% charcoal stripped serum and incubated further for 24 hours. Thereafter 250 ng TAT-GRE-Luc reporter plasmid and 25 ng β-galactosidase expression vector were transiently transfected into the cells, which were then incubated for an additional 48 hours. After the incubation period, the medium were replaced with phenol red-free, serum-free media and the cells were stimulated for 8 hours with vehicle (EtOH) or 100 nM PR-specific agonist, R5020. The cells were harvested, with luciferase and β-galactosidase assays, as well as Braford assays, were performed. The data was normalised for transfection efficiency and cell number by expressing luciferase (luc) activity relative to β-galactosidase activity and total protein. The graph shows pooled results from two independent experiments performed in triplicate, which is presented as fold induction relative to vehicle (EtOH) control.

Figure 5.3 shows that upon R5020 treatment, a small but not significant increase in PRE-reporter activity is seen (1.4 fold relative to vehicle). This result may suggest that low amounts of PR protein are expressed in LβT2 cells. However, more experiments are required to establish whether functional PR is expressed at high enough levels to elicit a significant PR-mediated response. Previous studies have shown the presence and a role for the PR in mediating LβT2 responses (Thackray *et al.,* 2006; Thackray *et al.,* 2009; An *et al.,* 2009). Yet it must be noted that some of these studies performed in the LβT2 cell line have used

exogenously expressed PR protein (Thackray *et al.,* 2009), suggesting that there are only low levels of PR present in this cell model.

5.2 The effect of hormone and combination treatments thereof on PR-B mRNA levels

Although no statistical significant levels of functional PR protein could be detected (Figures 5.2 and 5.3), *PR*-B mRNA is expressed in the LβT2 cell line (Figure 5.1). Additionally, as mentioned above, there is evidence for crosstalk between GnRHR and PR/ER/GR signalling pathways (An *et al.,* 2009; Chen *et al.,* 2009; Kotitschke *et al.,* 2009). In order to determine whether these pathways regulate *PR*-B mRNA expression, experiments were designed to assess the effect of the test compounds on endogenous LβT2 *PR-B* mRNA expression.

LβT2 cells were primed for 48 hours with or without 0.2 nM E2 to assess the effect of E2 priming on PR-mediated signalling. Thereafter the cells were treated for 8 hours with vehicle or test compounds; Dex, P4 GnRH and combinations thereof at saturating concentrations (100 nM). After ligand incubations, samples were harvested and RNA was extracted (Figure 9.6), followed by subsequent cDNA conversions. Quantitative real-time PCR with *PR*-B-specific primers was used to assess the transcriptional effects of hormones in regulating endogenous *PR-B* mRNA levels in LβT2 cells.

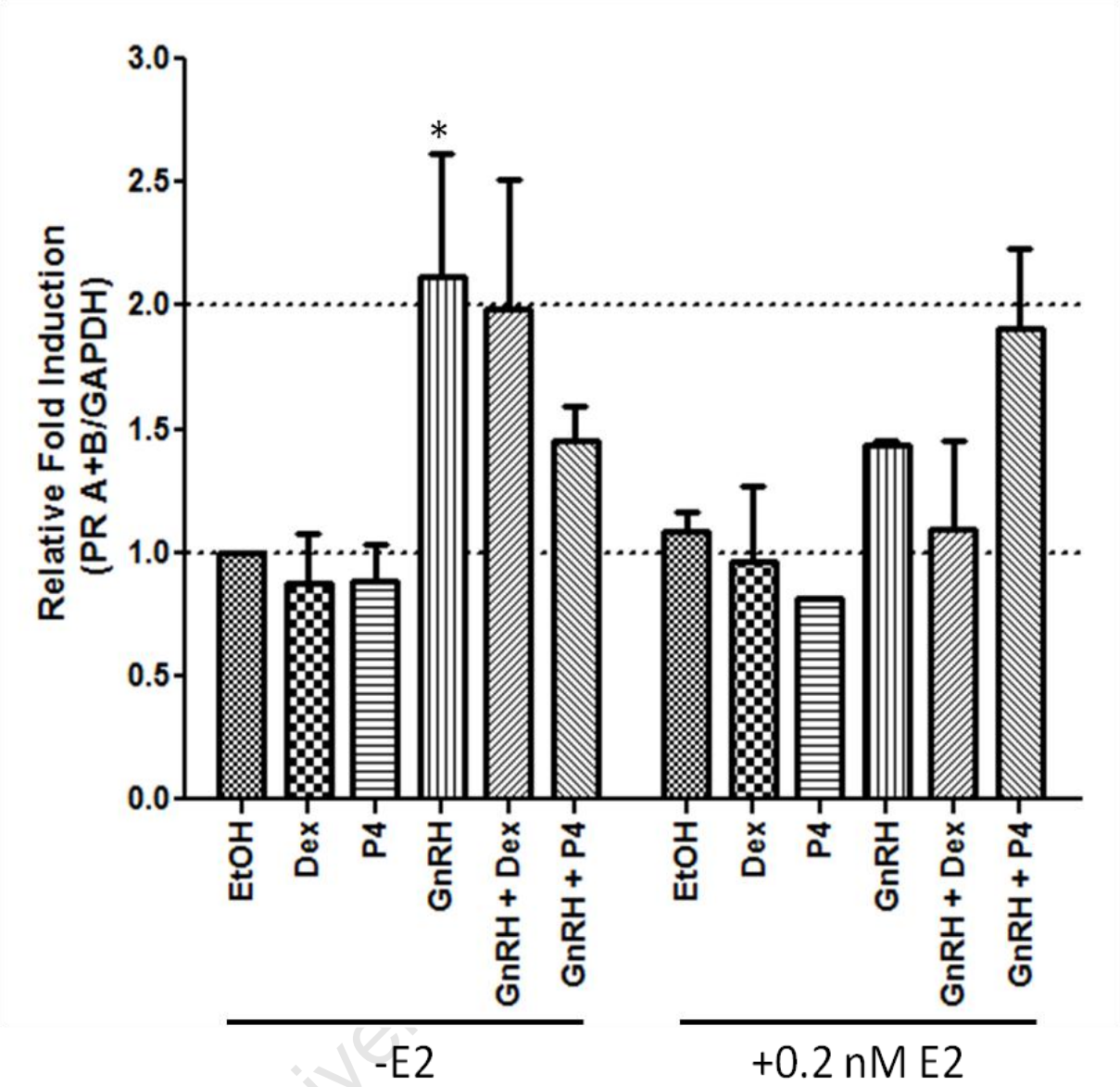


Figure 5.4: PRβ mRNA expression appears to be up-regulated in response to GnRH, but not by Dex, P4 or E2 treatments in LβT2 cells. LβT2 cells were plated into 12-well plates in DMEM supplemented with 10% serum. After a 24 hour incubation period, the cells were treated with or without 0.2 nM E2 in Phenol red-free media supplemented with 10% charcoal stripped serum. After 48 hours, the cells were treated with 100 nM vehicle (EtOH) or with the appropriate ligands and combinations thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting and subsequent RNA extractions. cDNA was generated for all mRNA sample sets and was further analysed using PR-B-specific primers and quantitative real-time PCR (rotogene machine; Rotogene-6000). Endogenous PR-B expression (Figure 11.8.1) was normalised to endogenous GAPDH expression (Figure 11.6.1), and was represented as fold induction relative to vehicle (EtOH). The graph shows pooled results for several independent experiments (n=4 for all samples without E2, n=3 for all E2 containing samples, and n=1 for +E2 P4 sample). Stars represent a significant ($P < 0.05$

= *) difference when compared to untreated control (-E2 EtOH), defined by a non-parametric, two-way ANOVA with a Bonferroni post test.

Figure 5.4 suggests that *PR*-B mRNA expression is regulated in a ligand-selective manner. Treatment with Dex or P4 showed no significant effects on mRNA levels (0.9 fold relative to vehicle). Current literature suggests a suppressive role for P4 in regulating *PR*-B expression in primary pituitary tissue (Graham and Clarke, 1997), yet this is in contrast with the current data (Figure 5.4). However, it must be noted that primary pituitary cells are composed of a number of different cell types, which may explain the differences in responses seen. No literature was found regarding the effects of P4 on endogenous PR mRNA expression in the LβT2 cell line. Interestingly GnRH treatment did significantly up-regulate endogenous *PR-B* mRNA expression (2 fold increase relative to vehicle).

Co-treatment with GnRH plus Dex on *PR*-B mRNA expression appeared to elicit a similar increase (2 fold increase) as seen with GnRH treatment alone, while co-treatment with GnRH plus P4 appeared to increase relative *PR*-B mRNA expression in LβT2 cells by 1.5 fold (Figure 5.4). These effects may be accounted for through the actions of GnRH treatment alone, as single treatments with Dex or P4 had no apparent effect on *PR*-B mRNA expression. However, it is possible that P4 is able to represses the GnRH response slightly when administered together, although further experiments are required to establish whether this result is significant. These combination treatments do not reveal any additive or synergistic signalling properties by these hormones in regulating endogenous *PR-B* mRNA expression in LβT2 cells, suggesting that potential crosstalk pathways between the GnRHR and the GR or PR may not regulate *PR*-B mRNA expression. It appears that GnRH treatment is the only treatment that significantly increases endogenous LβT2 *PR*-B mRNA expression (Figure 5.4).

E2 priming for 48 hours with 0.2 nM E2 resulted in no significant variation on endogenous *PR*-B mRNA expression in LβT2 cells (1.1 fold) as compared to un-primed vehicle (Figure 5.4). This result is consistent with the literature showing that E2 priming has no effect on *PR* expression in LβT2 cells (Turgeon, and Waring, 2006). Yet this treatment has been effective

in up-regulating *PR* mRNA levels in primary mouse and rat pituitary cells (Turgeon and Waring, 2006). Therefore, in general it may be considered that primary cells respond differently to immortalised LβT2 cell lines. Stimulation with Dex or P4 on +E2 primed LβT2 cells show no significant differences when compared to the –E2 primed data set (0.9 and 0.8 fold relative to vehicle respectively) (Figure 5.4). However the +E2 P4 treatment is representative of one biological repeat, and therefore cannot be statistically analysed. Therefore further repeats are required to determine the ligand-induced effects of P4 on E2 primed LβT2 cells.

Treatment with GnRH on E2 primed LβT2 cells appeared to increase relative *PR*-B mRNA expression (1.5 fold relative to vehicle). However, this appears to be a reduced response when compared to GnRH treatment on the un-primed LβT2 cells (2 fold relative increase) (Figure 5.4). It may be that E2 treatment dampens the GnRH response yet the experiment only represents a small data set, and further experiments are required to determine if this change in *PR*-B mRNA expression is significant.

Co-incubation with GnRH plus Dex on E2 primed LβT2 cells resulted in no significant differences compared to vehicle (1.1 fold), unlike the apparent 2-fold increase obtained with Dex plus GnRH treatments on un-primed samples. Taken together with the results for GnRH in the presence and absence of E2 priming strengthens the hypothesis that E2 priming may modulate ligand-dependent responses. Co-treatment with GnRH and P4 appeared to increase relative fold expression by 1.9 fold (Figure 5.4), which is a slightly higher response when compared to the un-primed E2 sample set.

Taken together, no effects were seen on LβT2 *PR*-B mRNA expression with E2 treatment alone, which is in agreement with current LβT2 literature (Turgeon and Waring, 2006). GnRH was the only hormonal treatment resulting in a significant increase in *PR-B* expression. Consistent with this, Dex/P4 plus GnRH co-incubations showed no additive or synergistic properties, suggesting that treatment with GnRH alone is responsible for the induction of endogenous *PR*-B mRNA expression in LβT2 cells. The result of hormone combination treatments on endogenous LβT2 *PR*-B mRNA expression suggests that there is no crosstalk signalling between PR/GR and GnRHR to regulate *PR-B* mRNA expression in LβT2 cells. However the apparent, but statistically insignificant effect of E2 priming on the GnRH and

GnRH plus Dex responses suggest possible crosstalk between the ER and GnRHR in regulating or modulating ERα mRNA expression.

5.3 Endogenous PR-B protein levels are not detected in response to GnRH treatment

Although no detectable PR protein was seen under basal conditions (Figure 5.2), GnRH appeared to up-regulate endogenous *PR*-B mRNA expression (Figure 5.4). Although basal PR protein could not be detected, it may be detectable after GnRH treatments. Thus PR-B protein levels may be influenced in responses to ligand treatments. To assess PR protein levels in response to ligand treatments and combinations thereof, LβT2 cells were primed for 48 hours with or without 0.2 nM E2. Thereafter the cells were treated for 8 hours with vehicle or test compounds: Dex, P4, GnRH and combinations thereof, at saturating concentrations (100 nM). After ligand incubations, samples were harvested, and resolved using western blotting techniques, and probed with a specific antibody raised against PR-B protein to assess PR-B protein levels in response to ligand treatments.

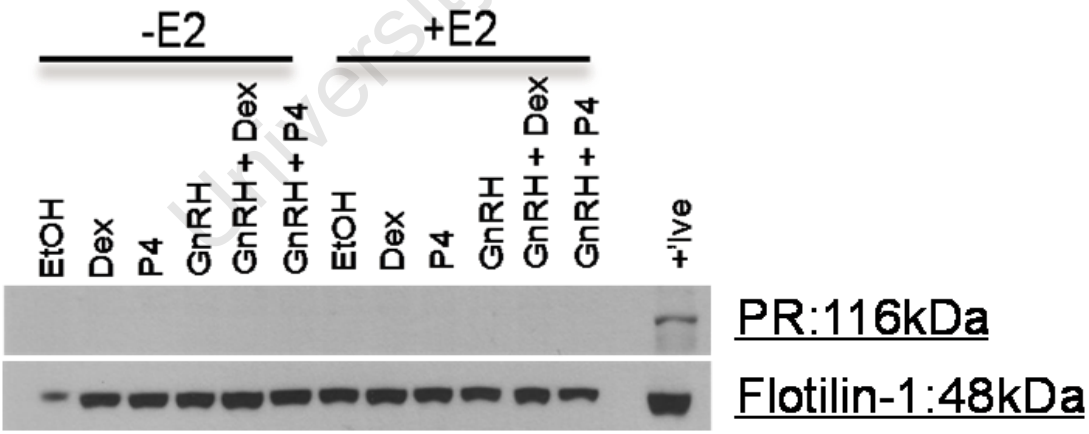


Figure 5.5: PR-B protein is not detectable under all conditions in LβT2 cells by Western blotting. LβT2 cells were plated into 12-well plates in DMEM supplemented with 10% serum. After a 24 hour incubation, the cells were treated with or without 0.2 nM E2 in phenol red-free media supplemented with 10% charcoal stripped serum. After 48hours, the cells were treated with 100 nM of the appropriate ligands and combination thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting in 50 µl SDS sample application buffer. Equal amounts (10

µl) LβT2 whole cell lysates were separated on a 8% SDS denaturing polyacrylamide gel, transferred to a nitrocellulose membrane and probed with PR-B-specific and Flotilin-1-specific (Loading control) primary polyclonal antibodies. Anti-rabbit-HRP secondary antibodies were used for detection of PR-B primary antibody, while anti-mouse-HRP secondary antibodies were used for detection of Fotilin-1 primary antibody. Signals were visualised with Amersham Chemiluminescence. PR-B and Flotilin-1 proteins were observed at 116 kDa and 48 kDa, respectively.

Similar to the results obtained in Figure 5.2, no detectable levels of PR-B could be seen under basal conditions, and remain undetectable under all ligand conditions (Figure 5.5). Furthermore, treatment with GnRH had no effect on detectable PR protein levels. Priming with E2 was also not sufficient to up-regulate PR protein, which is consistent with a previous LβT2 study regarding the effects of E2 priming on PR expression and translation (Turgeon and Waring, 2006). However, 8 hour ligand treatments may have resulted in insufficient time to allow for protein translation, even though the up-regulation of mRNA levels could already be detected after 8 hours (Figure 5.4). Therefore, additional samples should be collected at later time points, after hormone treatments, to confirm any delayed protein translation.

Taken together, no detectable levels of PR-B protein could be seen in un-stimulated or stimulated LβT2 cells by western blotting (Figure 5.5).

5.4 Endogenous PR protein cannot be up-regulated to induce PR-mediated transcription in LβT2 cells

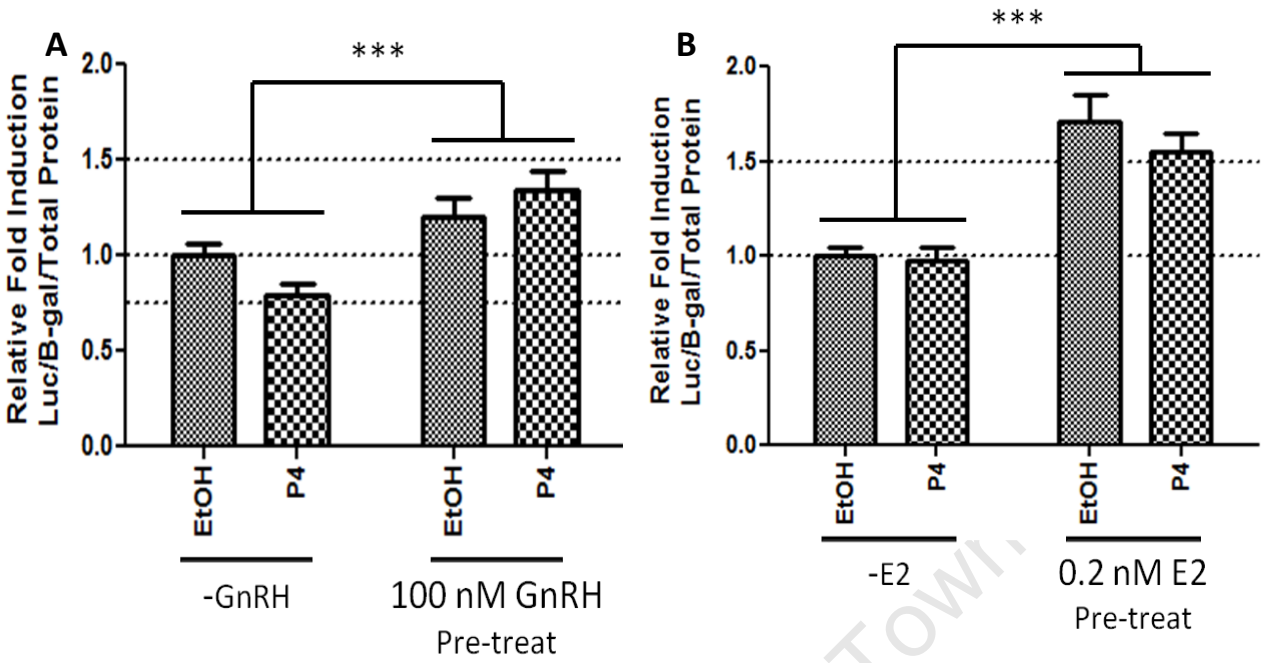
As shown previously, GnRH appeared to induce the up-regulation of *PR-B* mRNA (Figure 5.4), yet no detectable levels of PR protein were seen under the same conditions using Western blotting techniques (Figure 5.5). Eight hour incubation periods may have resulted in insufficient time to allow for sufficient protein translation, to the extent that protein would not be detectable by means of western blot analysis. Therefore a different approach was used to assess the role of GnRH in regulating PR protein.

In a previous study it was shown that GnRH mediates the rapid phosphorylation of PR, inducing the ligand-independent activation in LβT2 cells (An *et al.,* 2009). The *PR*-B mRNA expression data suggests that GnRH also up-regulates PR mRNA levels (Figure 5.4). Therefore attempts were made to assess the role of GnRH on PR activity on a PRE in the absence and presence of PR agonist (R5020) in LβT2 cells.

Assuming GnRH increases *PR*-B mRNA expression and hence protein expression, an increase in PRE-reporter activity would be expected with R5020 under GnRH pre-treatment. However, if GnRH also activates the PR in a ligand-independent manner, as An *et al.,* suggested, then GnRH would also increase basal PRE-reporter activity.

Additionally, the effects of 0.2 nM E2 priming revealed no significant changes in PR mRNA and protein levels (Figure 5.4 and Figure 5.5). This is consistent with current literature regarding LβT2 cells (Turgeon and Waring, 2006), but inconsistent with results obtained from primary mouse and rat pituitary cells, where it was shown that E2 priming over 3 days results in an increase in endogenous *PR* mRNA expression (Turgeon, and Waring, 2006).

Thus a TAT-GRE reporter-promoter assay was used to assess the transcriptional activity of PR protein in the absence and presence of agonist, in response to GnRH or E2 pre-treatments. A GRE reporter was used because the PR is able to act via a GRE, as the consensus sequence of a GRE is identical to that of a PRE. Therefore LβT2 cells transiently transfected with 250 ng TAT-GRE-luc reporter plasmid were pre-treated with and without GnRH or E2 for 24 hours prior to 8 hour stimulation with PR-specific agonist R5020 at saturating concentrations (100 nM).



Treatment (A)	Mean	Std. Dev.	Treatment (B)	Mean	Std. Dev.
EtOH	1.000	0.190	**EtOH**	1.000	0.112
R 5020	0.791	0.185	**R 5020**	0.976	0.170
(GnRH pre-treat) EtOH	1.201	0.297	**(E2 pre-treat) EtOH**	1.708	0.345
(GnRH pre-treat) R5020	1.340	0.304	**(E2 pre-treat) R5020**	1.550	0.243

Figure 5.6 (A-B): No PR-mediated transactivation seen on a PRE after GnRH (A) and E2 (B) pre-treatments in LβT2 cells. LβT2 cells were seeded into a 24-well plate in DMEM supplemented with 10% serum. After a 24 hour incubation period, the medium was replaced with phenol red-free media, supplemented with 10% charcoal stripped serum and incubated further for 24 hours. Thereafter 250 ng ERE-Luc reporter plasmid and 25 ng β-galactosidase expression vector were transiently transfected into the cells and were incubated with or without 100 nM GnRH (A) or 0.2 nM E2 (B) for an additional 24 hours. Thereafter, the medium was replaced with phenol red-free, serum-free media and the cells were the stimulated for 8 hours with vehicle (EtOH) or 100 nM PR-specific agonist, R5020. The cells were harvested, and luciferase, β-galactosidase and Brafords assays were performed. The data was normalised for transfection efficiency and cell number by expressing luciferase (luc) activity relative to β-galactosidase activity and total protein. The graph shows pooled results from three independent experiments, each performed in triplicate, which is presented as fold induction relative to vehicle (EtOH) control. Stars represent a significant ($P < 0.001$ = ***) defined by a non-parametric, two-way ANOVA with a Bonferroni post test.

Figure 5.6A shows the effects of GnRH pre-treatment on PRE/GRE-transcription in LβT2 cells. Upon 8 hour R5020 treatment alone it appears that a slight reduction in relative activity is seen on PRE-activity. This result suggests no functional PR protein is present in LβT2 cells under basal conditions, which is consistent with previous results obtained in this study (Figure 5.2 and Figure 5.5). Pre-treatments with GnRH alone significantly increased basal PRE-reporter activity to 1.2 fold relative to vehicle. On addition of 8 hour PR agonist (R5020) treatments, it appears that a slight increase in PRE-reporter activity is seen, relative to its internal control (GnRH pre-treatment plus EtOH), which may be due to GnRH up-regulating *PR*-B expression. However, this result is not statistically significant. Whether this increase in basal reporter activity is mediated by endogenous PR is unknown. Given the undetectable response to R5020, it is possible that the cells contain a mutated version of the PR that is unable to respond to P4 or R5020, but can be activated by GnRH, to increase transcription via binding to the PRE. This mechanism is possible, since An *et al.,* (2009) showed GnRH treatment mediates the site-specific phosphorylation of the PR protein to increase endogenous LβT2 fshβ mRNA expression (An *et al.,* 2009). On the other hand it is possible that the response observed in the current study is independent of the PR, and may either be mediated by GnRH activating some component(s) of basal transcription machinery present on the PRE, or GnRH ligand-independently activating the GR to drive GRE-reporter activity (Kotitschke *et al.,* 2009).

Considering the effects of E2 pre-treatment (Figure 5.6B), PR agonist (R5020) treatment alone revealed no difference compared to vehicle, which is consistent with the result that little to no functional PR is present in LβT2 cells (Figure 5.3). However the effect of 24 hour 0.2 nM E2 treatment showed a significant increase in basal transcriptional activity (1.7 fold relative to vehicle). However, PR agonist treatments on E2 primed LβT2s showed no apparent increase in PRE-mediated transcription (1.5 fold relative to vehicle). This result is consistent with *PR-B* expression data observed in the study (Figure 5.4), showing that E2 treatment does not up-regulate *PR*-B mRNA expression, as well as other studies in the LβT2 cell line (Turgeon and Waring, 2006), but inconsistent with data observed in primary pituitary cells (Turgeon and Waring, 2006).

Taken together, attempts to up-regulate functional PR protein and PRE-reporter activity in LβT2 cells though ligand pre-treatments was unsuccessful, suggesting that LβT2 cells have very low levels of functional classical PR-B.

5.5 mPRα does not mediate the effects of P4 in LβT2 cells

Recent studies have shown the presence of a novel mPR in mediating non-genomic P4 signalling in various tissue types (Zhu *et al.,* 2003). The present study has shown that in the LβT2 cell line grown in our laboratory, PR protein is undetectable by western blotting (Figure 5.2 and Figure 5.5). Endogenous *PR-B* mRNA expression is not significantly affected by treatments with Dex, P4, and E2 (Figure 5.4) and functional PR-B activity is not detectable on a PRE-luc reporter promoter assay (Figure 5.3). Nevertheless both GnRHR (Figure 3.2) and ERα (Figure 4.4) mRNA levels are regulated by P4 treatment, suggesting the presence of a functional PR. Thus it is possible that P4 signalling may be mediated through receptors besides the classical PR in LβT2 cells.

In light of this theory, an attempt was made to assess the presence of mPRα mRNA expression in the LβT2 cell line. LβT2 cells were cultivated under basal conditions, and cDNA samples were generated. *mPRα* mRNA was amplified using conventional PCR and a mPRα-primer pair (Addendum B, Table 10.1). cDNA from human endo-cervical cells (End-1) was used as a positive control as previous work from the Hapgood lab has shown mPRα mRNA expression in this cell line (Jaravaza, 2009). Note that these End-1 cells were found, by conventional PCR, to express mPRα and GR, but not PR (A+B) mRNA (Addendum A, Figure 9.2), while no PR-A or PR-B protein was detected by Western blotting (Adendum A, Figure 9.3)

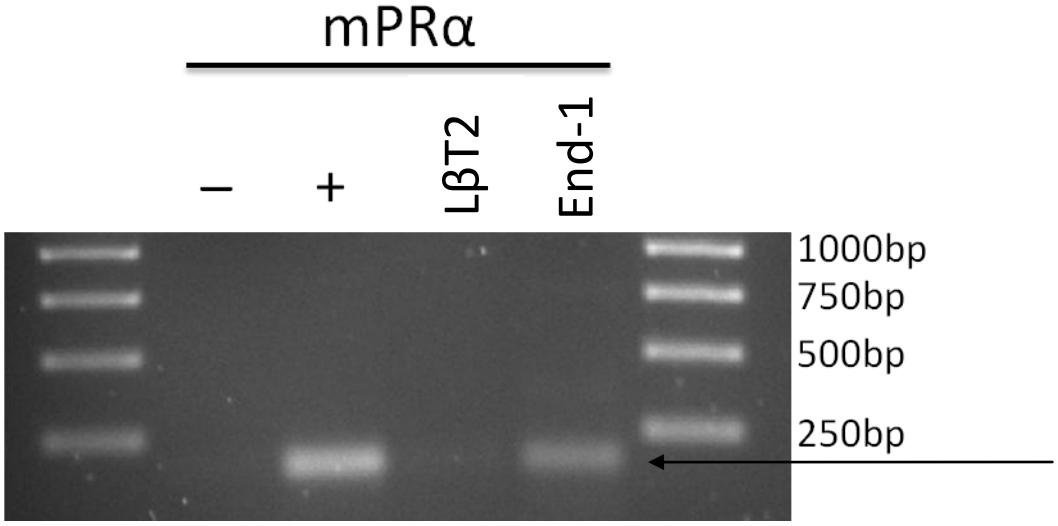


Figure 5.7: mPR mRNA is not expressed under basal conditions in LβT2 cells. LβT2 cells were seeded into 6-well plates in DMEM supplemented with 10% serum, and were left to grow to 70% confluency under basal conditions. End1/E6E7 (End-1) cells were seeded into 12-well plates in KSF media,. Thereafter both cell lines were left to grow to 70% confluency under basal conditions. Thereafter End-1 and LβT2 cells were harvested and RNA extractions were performed, followed by a reverse transcriptase reaction to generate basal cDNA samples. Signals were amplified using conventional PCR with mPRα-specific primer pairs. An mPRα expression vector (pcDNA3.1/mPRα) was used as a positive control (+), and 1 µl dH_2O was used as the input for the no template control (-). PCR products were generated over 30 cycles, separated on a 2% agarose gel using electrophoresis, and visualised using ethidium bromide staining. Arrow indicates mPRα amplicon at 214 bp.

Figure 5.7 indicates that LβT2 cells do not express endogenous *mPRα* mRNA, suggesting that the effects of P4 on LβT2 cell responses are not mediated by mPRα. A positive signal at 241 bp is seen for the endo-cervical cell line (End-1) (Figure 5.7). Although human *mPRα* primers were used in the PCR reaction (Figure 5.7) on mouse LβT2 samples, NCBI mRNA sequence blast did show that the human *mPRα* primer pair used can anneal to mouse mPRα mRNA transcript (data not shown).

Taken together, the results show End-1 cells lack PR-A and PR-B mRNA and protein expression (Addendum A, Figures 9.2 and 9.3), yet mPRα mRNA is basally expressed in the

End-1 cell line. This suggests that mPRα may play an important role in mediating the effects of P4, as these cells have been shown to respond to P4 (Zhu *et al.,* 2003; Cai *et al.,* 2005; Karteris *et al.,* 2006) and progestins (Verhoog, 2010).

For a side study, mPRα exogenous expression and mPRα protein detection was optimised in light of the hypothesis that mPRα may play an important role in P4-mediated signalling in the End-1 cell line. Therefore several mPRα expression vectors were acquired (Krietsch *et al.,* 2006), to serve as a positive control in the investigation of mPRα protein levels in the End-1 cell line. mPRα expression vectors were subsequently cloned and sequenced, so that detailed plasmid maps could be constructed for future reference (Addendum D, Figures 12.1, 12.2 and 12.3). To optimize exogenous mPRα expression, End-1 cells were transfected with tagged mPRα-HA or untagged mPRα expression constructs and left to grow under basal conditions. Thereafter End-1 cell lysates were harvested (using multiple harvesting protocols, outline in methods Section 2.13) and probed for mPRα protein using Western blot techniques and anti-HA- (Addendum A, Figure 9.4) or anti-human/mouse mPRα-specific primary antibodies (Addendum A, Figure 9.5). A summary of exogenous mPRα protein expression is shown in Addendum A, Table 9.1. An 80 kDa mPRα dimer or a 40 kDa mPRα monomer (Krietsch *et al.,* 2006) were not detected using DEAE or Fugene transfection protocols. Transfection controls were incorporated for all experiments (pCMV-HA-human GR) and yielded positive protein expression under all conditions, indicating transfection conditions used were appropriate. This result may suggest improper protein folding and/or membrane localisation of the mPRα protein in the End-1 cell line. To address this hypothesis, mPRα over-expression was attemped in the LβT2 cell line, on the account of its endogenous GPCR (GnRHR) expression (Navratil *et al.,* 2009). Exogenous mPRα protein expression appeared unsuccessful under all transfection conditions (Addendum A, Figure 9.4). It was concluded that the mPRα expression vectors must be the root of the problem, as a result of no differences between mock (-mPRα) and transiently transfected (+mPRα) End-1 and LβT2 cells (Addendum A, Figure 9.4).Therefore any attempt to characterise End-1 cells for endogenous mPRα protein expression proved unsuccessful as no over-expressed mPRα protein controls could be generated.

On a separate issue, it should be noted that a number of non-specifics bands were seen when using the anti-mPRα anti body (Addendum A, Figure 9.5). However this is most likely due to a high exposure of western blot development in assessing mPRα protein levels.

In conclusion, LβT2 cells show no detectable levels of functionally active PR protein using Western blotting (Figure 5.2), and no detectable levels of endogenous *mPRα* mRNA expression using conventional PCR (Figure 5.7). This result suggests that the mPRα is not involved in mediating P4 responses in the LβT2 cell line.

CHAPTER 6

RESULTS AND DISCUSSION

6. Regulation of GRα expression and function in LβT2 responses

As mentioned earlier, LβT2 genes have been shown to be regulated by the GR via GR ligand-dependent and GR ligand-independent mechanisms (Kotitschke *et al.,* 2009), with previous results showing crosstalk between the GR and GnRHR signalling pathways.

It is apparent that GnRH signalling effects *ERα* (Figure 4.4) and *PR-B* (Figure 5.4) mRNA expression in LβT2 cells, and that GR and GnRHR crosstalk regulates GnRHR mRNA expression in LβT2 cells (Figure 3.2). In addition, a novel and interesting effect of E2 priming appeared to modulate Dex-mediated responses on GnRHR (Figure 3.2), and possibly also *ERα* mRNA expression (Figure 4.4). Therefore the effects of Dex, P4, E2, GnRH and combinations thereof on GR expression was investigated in LβT2 cells.

6.1 Characterisation of endogenous GRα in LβT2 cells

Before assessing a role for hormone signalling in regulating *GRα* mRNA expression, untreated LβT2 cells were first used to characterise endogenous *GRα* mRNA levels under basal conditions. LβT2 cells were cultivated under basal conditions, and harvested. Subsequent RNA extractions and cDNA conversions were performed to generate LβT2 cDNA. Thereafter conventional PCR with intron spanning GRα-specific primers was used to assess basal *GRα* mRNA expression in LβT2 cells.

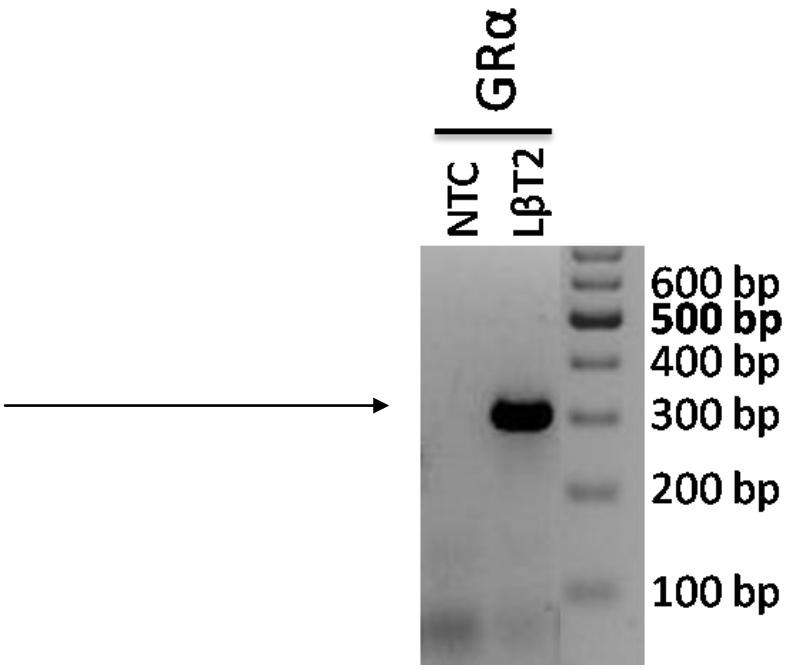


Figure 6.1: Endogenous GRα mRNA is basally expressed in LβT2 cells. LβT2 cells were seeded into 6-well plates in DMEM supplemented with 10% serum, and were left to grow to 70% confluency under basal conditions. Thereafter the cells were harvested and RNA extractions were performed, followed by a reverse transcriptase reaction to generate basal LβT2 cDNA. Signals were amplified using conventional PCR with GRα-specific primer pairs. PCR products were generated over 35 PCR cycles and separated on a 2% agarose gel using electrophoreses, and visualised with ethidium bromide staining. Arrow indicates GRα amplicon at 299 bp. NTC defines the no template control.

From Figure 6.1 it is clear that *GRα* mRNA is expressed under basal conditions in LβT2 cells, in agreement with the current literature showing endogenous LβT2 *GRα* expression (Kotitschke *et al.,* 2009; Nicolaides *et al.,* 2010). Having shown the presence of GRα mRNA in LβT2s cells, it was next determined whether LβT2 cells express detectable levels of GRα protein. Crude cell lysates were generated from cultivated LβT2 cells and GR protein levels were investigated using western blotting with specific anti-GRα antibodies. COS-7 cells over-expressing hGRα-HA were harvested and the extracts electrophoresed alongside LβT2 lysates on the western blot to serve as a positive control.

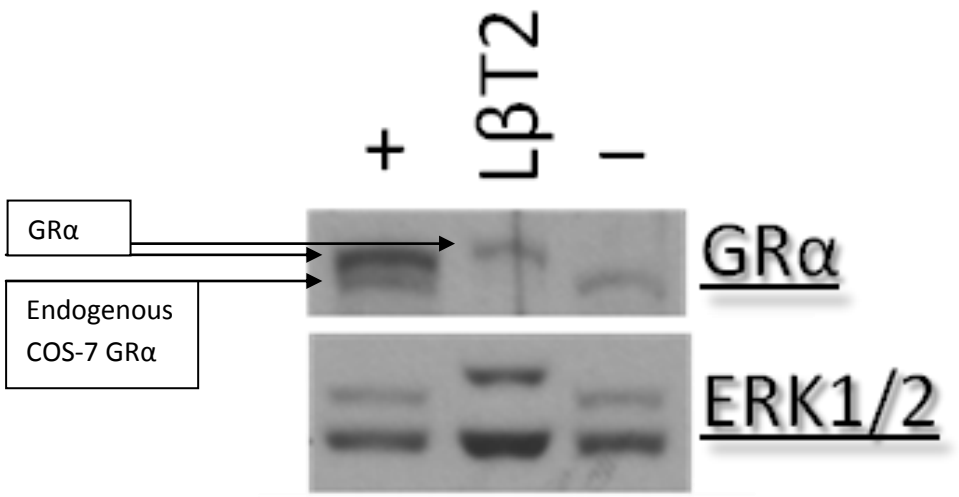


Figure 6.2: Endogenous GRα protein is expressed under basal conditions in LβT2 cells. COS-7 cells were seeded into 6-well plates in DMEM supplemented with 10 % serum. After a 24 hour incubation, the cells were either transiently transfected with 1 µg of pGMV-HA-hGR expression vector (Positive control) (+) or left to grown under basal conditions (negative control) (-). LβT2 cells were seeded into a 6-well plate in DMEM supplemented with 10% serum, and left to grow under basal conditions. After all cells had reached a 70% confluency (at least 24 hours after transfection), the COS-7 cells and untreated LβT2 cells were harvested in 50 µl SDS sample application buffer. Equal volumes (10 µl) of all lysates were separated on 8% SDS polyacrylamide gel, transferred to a nitrocellulose membrane and probed with GRα-specific or ERK1/2-specific (Loading control) primary polyclonal antibodies. Anti-rabbit-HRP secondary antibodies were used for detection of both GRα and ER1/2 primary antibodies. Signals were visualised with Amersham Chemiluminescence. The top arrows indicate the GRα protein observed at 95 kDa, with the second arrow indicates a non-specific band (N/S) at 94 kDa. ERK1/2 proteins are seen at 42 kDa and 44 kDa, respectively.

From Figure 6.2 it is evident that GRα protein is present under basal conditions in LβT2 cells, in agreement with the current literature showing expression of GRα protein in LβT2 cells (Kotitschke *et al.,* 2009). It must be noted that a non-specific band was seen in the COS-7 positive and negative control cell lysates. This non-specific band (as indicated by the arrow in Figure 6.2) was seen to run at a slightly lower molecular weight compared to the over-expressed GRα protein signal, and is most like endogenous COS-7 GRα protein. These

differences in protein migration through the SDS polyacrylamide gel may be a result of cell-specific differences between COS-7 and LβT2 cells

Taken together, Figures 6.1 and 6.2 illustrate that GRα mRNA is basally expressed and translated into GR protein. To test whether this GR protein is functionally active in LβT2 cells and will drive GRE promoter-reporter transactivation upon agonist treatment, LβT2 cells were transiently transfected with a TAT-GRE-luc reporter plasmid, and treated with GR agonist (Dex) at saturating concentrations (100 nM) for 8 hours.

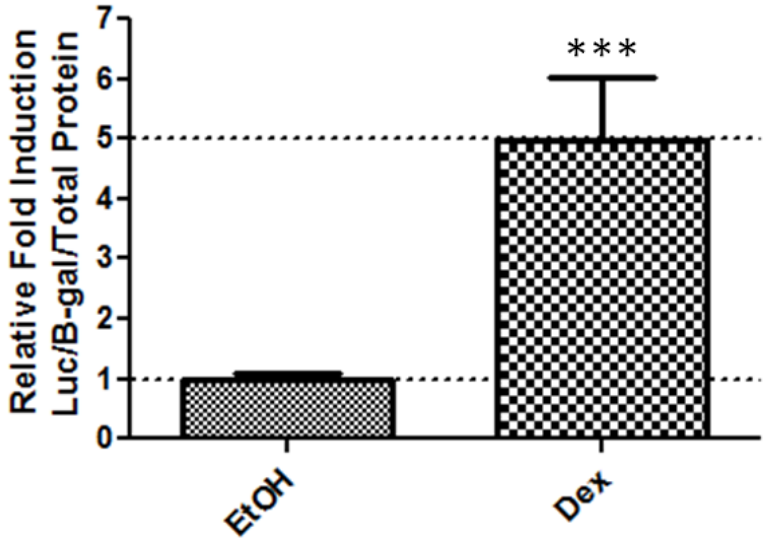


Figure 6.3: Functional reporter assay shows endogenous GRE-mediated transcription in LβT2 cells. LβT2 cells were seeded into a 24-well plate in DMEM supplemented with 10% serum. After a 24 hour incubation period, the medium was replaced with phenol red-free media, supplemented with 10% charcoal stripped serum and incubated further for 24 hours. Thereafter 250 ng TAT-GRE-Luc reporter plasmid and 25 ng β-galactosidase expression vector were transiently transfected into the cells, which were then incubated for an additional 48 hours. Thereafter, the medium was replaced with phenol red-free, serum-free media and the cells were the stimulated for 8 hours with vehicle (EtOH) or 100 nM GR-specific agonist, Dex. Thereafter, the cells were harvested, and luciferase, β-galactosidase and Brafords assays were performed. The data was normalised for transfection efficiency and cell number by expressing luciferase (luc) activity relative to β-galactosidase activity and total protein. This graphs shows pooled results from two independent experiments, each performed in triplicate, which is presented as fold induction relative to vehicle (EtOH) control. Stars

represent a significant (P < 0.001 = ***) difference when compared to untreated control, defined by a two-tailed T-test

Figure 6.3 shows a 6 fold increase in relative TAT-GRE-reporter-activity in response to 100 nM GR agonist (Dex) treatment. This is consistent with the genomic roles of Dex in activating the GR to drive GRE-promoter expression (Nicolaides *et al.,* 2010).

6.2 GRα mRNA expression is ligand-dependently regulated in response to hormone treatments in LβT2 cells

As the *GRα* is endogenously expressed in LβT2 cells (Figures 15 and 16), there is a potential for the *GRα* gene to be transcriptionally regulated in response to ligand treatments. Additionally, as mentioned earlier, recent evidence has shown that crosstalk mechanisms occur between the GRα and other receptors (Kotitschke *et al.,* 2009; Chen *et al.,* 2009; An *et al.,* 2009). Therefore to determine whether these crosstalk pathways (ER/PR/GR and GnRHR) feedback to regulate *GRα* mRNA expression, experiments were designed to assess the effect of appropriate test compounds (Dex, P4, GnRH and combinations thereof) on endogenous LβT2 GR*α* mRNA expression.

LβT2 cells were pre-treated with or without 0.2 nM E2 for 48 hours, as well as an 8 hour treatment at saturating concentrations (100 nM) of Dex, P4, GnRH and combinations thereof. Thereafter cells were harvested and RNA was extracted (Figure 9.6), followed by subsequent cDNA conversion. Quantitative real-time PCR with GRα-specific primers were used to assess the transcriptional effects of Dex, P4, GnRH, E2 and combinations thereof in regulating endogenous *GRα* mRNA levels in LβT2 cells.

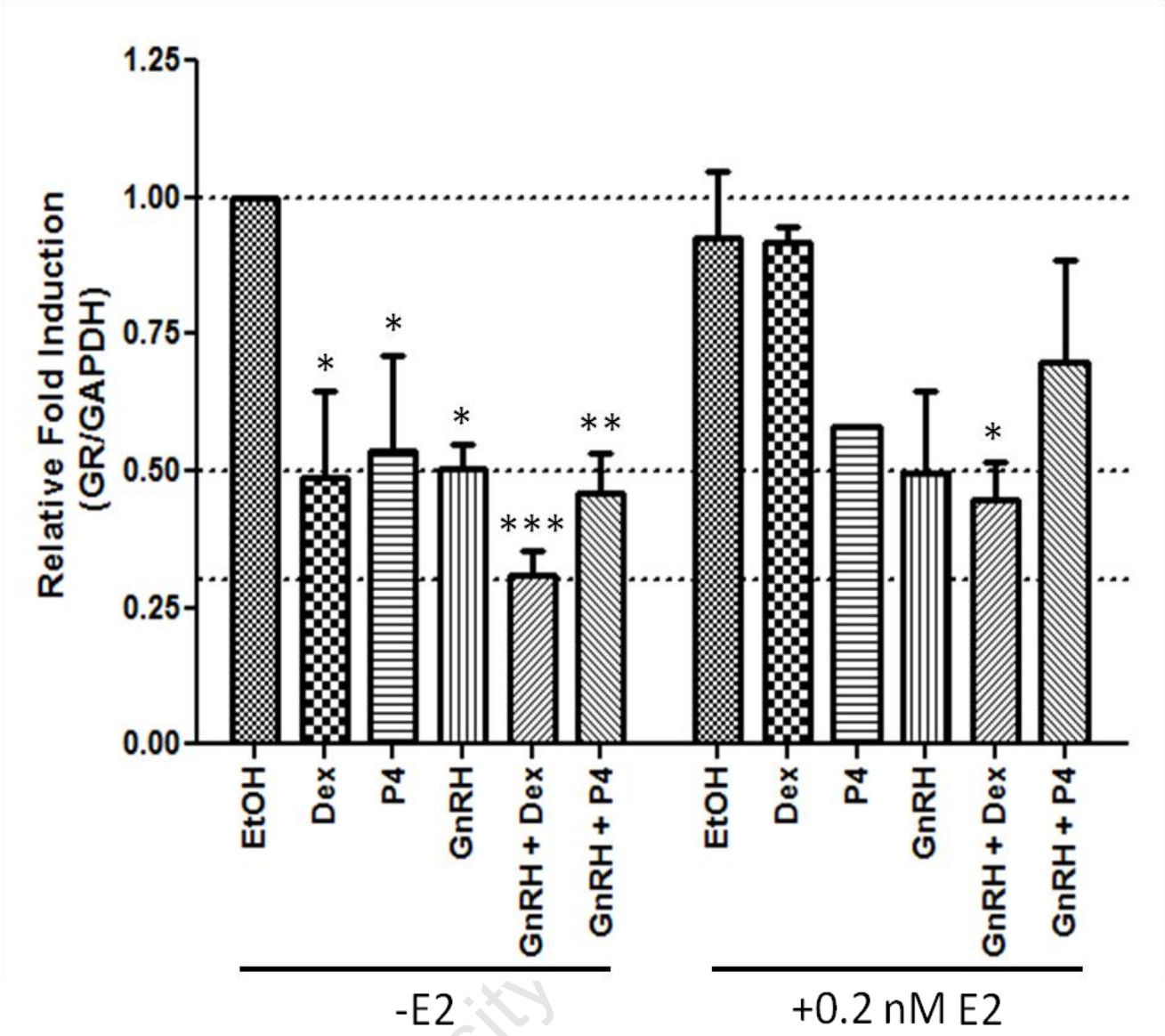


Figure 6.4: GR mRNA expression is significantly suppressed in a ligand-dependent manner in LβT2 cells. LβT2 cells were plated into 12-well plates in DMEM supplemented with 10% serum. After a 24 hour incubation, the cells were treated with or without 0.2 nM E2 in phenol red-free media supplemented with 10% charcoal stripped serum. After 48 hours, the cells were treated with 100 nM appropriate ligand and combination thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting and subsequent RNA extractions. cDNA was generated for all mRNA sample sets, and was further analysed with GRα-specific primers and quantitative real-time PCR (Rotogene machine; Rotogene-6000). Endogenous GRα mRNA expression (Figure 11.9.1) was normalised to endogenous GAPDH expression (Figure 11.6.1), and was represented as fold induction relative to vehicle (EtOH). The graph shows pooled results for several independent experiments (n=4 for all samples without E2, n=3 for all E2 containing samples, and n=1 for +E2 P4 sample). Stars represent a significant ($P < 0.05$ = * $P < 0.01$ = **) difference when compared to untreated control (-E2 EtOH), defined by a non-parametric, two-way ANOVA with Bonferroni post test.

From Figure 6.4, Dex treatment significantly down-regulated *GRα* mRNA expression to 0.5 fold relative to vehicle, which is consistent with the current literature showing that GCs down-regulate GRα mRNA expression in most target cell lines (Burnstein *et al.,* 1990; De Silva *et al.,* 1993; Freeman *et al.,* 2004). Additionally a study has shown a correlation between Dex treatments and an increase in GR turn-over (Avenant *et al.,* 2010). Therefore this result supports the hypothesis that GCs reduce relative levels of GR mRNA and protein expression as a result of a feedback mechanism in response to GR-mediated signalling. Treatment with P4 resulted in a similar significant repressive trend on LβT2 *GRα* mRNA expression, reducing mRNA levels to 0.55 fold relative to vehicle (-E2 EtOH). Because neither PR (A+ B) nor mPRα protein was previously detected in the LβT2 cell line, this raises the question of how the effects of P4 are mediated. This result suggests the possibility that the GR may be responsible for the P4-mediated down-regulation of *GR*α mRNA expression via the partial agonist properties of P4 towards the GR (Ronacher *et al.,* 2009).

Stimulation with GnRH resulted in a similar significant repressive trend as compared to Dex treatment (0.5 fold relative to vehicle). This result suggests a number of possibilities. One explanation is that GnRH is acting via the unliganded GR, as suggested by Kotitschke *et al.,* Therefore the ligand-independent activation of the GR by GnRH may regulate endogenous *GRα* mRNA expression through *cis*-elements in the GR gene promoter, possibly to serve as a signalling feedback mechanism in LβT2 cells. Another explanation could suggest that GnRH acting via GnRHR is able to regulate the GR-promoter activity via kinase signalling cascades involved in GnRHR signalling (PKA, PKC or MAPK) to regulate downstream transcription factors like AP-1 (Kotitschke *et al.,* 2009). A final explanation may involve a combination of the two mechanisms. Yet a precise mechanism cannot be established with the current data available, and further experiments are required to assess these hypothesised mechanisms.

Co-stimulation with Dex plus GnRH further reduced *GRα* mRNA levels to 0.3 relative fold induction, compared to the 0.5 fold reduction with Dex or GnRH treatment alone. This novel finding reveals that the repressive properties of Dex and GnRH signalling may act in concert to repress *GR*α expression (Figure 6.4). This result may be representative of two separate pathways, as the Dex plus GnRH response is not additive or synergistic in nature. It is

possible that the effects of Dex are mediated through the ligand-dependent activation of the GR, while the effects of GnRH may be a result of GnRHR-mediated signalling. However, further experiments would be required to assess the statistical significance of this result, and elucidate a mechanism responsible for mediating this response.

Co-stimulation with P4 plus GnRH elicited a similar response as compared to GnRH treatment alone, reducing *GRα* mRNA levels to 0.45 fold relative to vehicle (Figure 6.4). However no additive properties were observed with P4 plus GnRH treatment. This result appears to be different to the effects of Dex plus GnRH treatment, suggesting that the effects of P4 are not mediated by the GR.

Taken together, the un-primed results in Figure 6.4 show a potential for Dex and GnRH signalling pathways to be functionally integrated in regulating LβT2 *GRα* mRNA expression, however further experiments are required to assess this hypothesis. What can be said is that Dex and GnRH feedback signalling must exist to modulate GRα mRNA expression in LβT2 cells.

Priming with 0.2 nM E2 for 48 hours resulted in no significant change in basal *GRα* mRNA expression in LβT2 cells (0.9 fold relative to vehicle) (Figure 6.4). Stimulation with Dex on E2 primed LβT2 cells appeared to show a novel abolishment of Dex-induced repression of *GRα* mRNA expression when compared to un-primed Dex treatment, with mRNA levels remaining unchanged at 0.9 fold relative to E2 primed vehicle (+E2 EtOH) (Figure 6.4). This result suggests a switch from a repressive role for Dex signalling in regulating *GRα* mRNA, to no effect on the addition of 48 hour E2 priming. This result appears to support ER and GR crosstalk (Figures 3.2 and 4.4), suggesting that prolonged low doses of E2 may influence the expression of GR regulated genes in response to GCs. This response to E2 may occur indirectly through ER-mediated changes in GR phosphorylation at Ser-211, as seen with the E2-induced up-regulation of protein phophatase 5 (PP5) expression in MCF-7 cells (Zhang *et al.,* 2009).

Treatment with P4 on E2 primed LβT2 cells resulted in a similar trend to that obtained with P4 alone, i.e. repression of *GRα* mRNA levels to 0.6 fold relative to vehicle (Figure 6.4). However the result is representative of one biological repeat and additional experiments are

required to determine the significance of E2 priming on P4 responses in LβT2 cells. However it appears that low doses of E2 treatment do not affect the repressive effect of P4 regulating *GRα* mRNA expression. This result argues against the hypothesis that P4 signalling acts via the GR, as the effects of E2 priming were only influential in abolishing Dex-induced repression and not P4-induced repression. This suggests that Dex and P4 signalling is mediated by two different receptor pathways to regulate *GR*α mRNA levels.

Treatments with GnRH on E2 primed LβT2 cells appeared to have a similar repressive trend in regulating *GRα* mRNA expression when compared to the un-primed sample set (Figure 6.4), with relative *GRα* mRNA levels dropping to 0.5 fold expression compared to vehicle (Figure 6.4). Thus E2 priming does not affect GnRH-mediated signalling in regulating LβT2 *GRα* mRNA expression. Co-stimulation with Dex plus GnRH on E2 primed LβT2 cells reduced *GRα* mRNA levels to 0.45 fold relative to vehicle (Figure 6.4). Although repression is still observed upon Dex plus GnRH co-treatment, the combined repressive effects of Dex and GnRH appear to be reduced when compared to the un-primed Dex plus GnRH sample set. This suggests that the response may be representative of GnRH action alone, as E2 priming was able to reduce Dex-mediated repression (Figure 6.4). Further experiments are required to establish whether this change in Dex-response between E2 primed and un-primed LβT2 cells is statistically significant. Stimulation with P4 plus GnRH co-treatment reduced *GRα* mRNA levels to 0.7 fold in relative expression (Figure 6.4). Once again this suggests that P4 does not act via the GR and that E2 priming does not influence P4 or GnRH-mediated regulation of *GRα* mRNA expression in LβT2 cells.

In conclusion, Figure 6.4 shows that endogenous LβT2 *GRα* mRNA is transcriptionally repressed in a ligand-dependent manner, upon Dex, P4 and GnRH treatments alone, but not with E2 priming alone. The finding that GR mRNA repression in the presence of Dex plus GnRH appears to be greater than that observed with each ligand alone, could suggest Dex-mediated and GnRH-mediated signalling are acting simultaneously. This would suggest GR and GnRHR crosstalk regulates GR levels. Additionally the novel result of E2 priming appearing to abolish Dex-mediated repression of *GR*α mRNA, may suggest GR and ER crosstalk may regulate GR levels. However, E2 priming did not appear to affect P4-mediated or GnRH-mediated *GR*α mRNA repression. An interesting observation was that responses

involving (GR-specific) Dex treatments seemed to be the only responses affected by E2 priming (Figures 3.2 and 4.4), while no effects were seen on GnRH or P4 signalling. The implications of this result suggest ER and GR signalling pathways may crosstalk in order to finely balance endocrine activity in the body. This further suggests that low doses of E2 may modulate GR-mediated activity, yet additional experiments are required to assess the involvement of the GR and ER in this response.

6.3 GRα protein levels are ligand-dependently regulated in response to hormone treatments in LβT2 cells.

As shown earlier, functional GR protein is present under basal conditions (Figure 6.2 and Figure 6.3). This study has shown GR mRNA levels are regulated in response to ligand treatments (Figure 6.4), suggesting ligand-dependent mechanisms regulate GR-mediated responses. Therefore the rationale was to determine whether observed changes in GRα mRNA levels mimicked the changes in protein levels. To measure varying GR protein levels, LβT2 cells were primed for 48 hours with or without 0.2 nM E2. Thereafter the cells were treated for 8 hours with vehicle or test compounds, Dex, P4 GnRH and combinations thereof at saturating concentrations (100 nM). Thereafter, samples were harvested, and resolved using western blotting, and probed with a specific antibody raised against GRα protein to assess GRα protein levels.

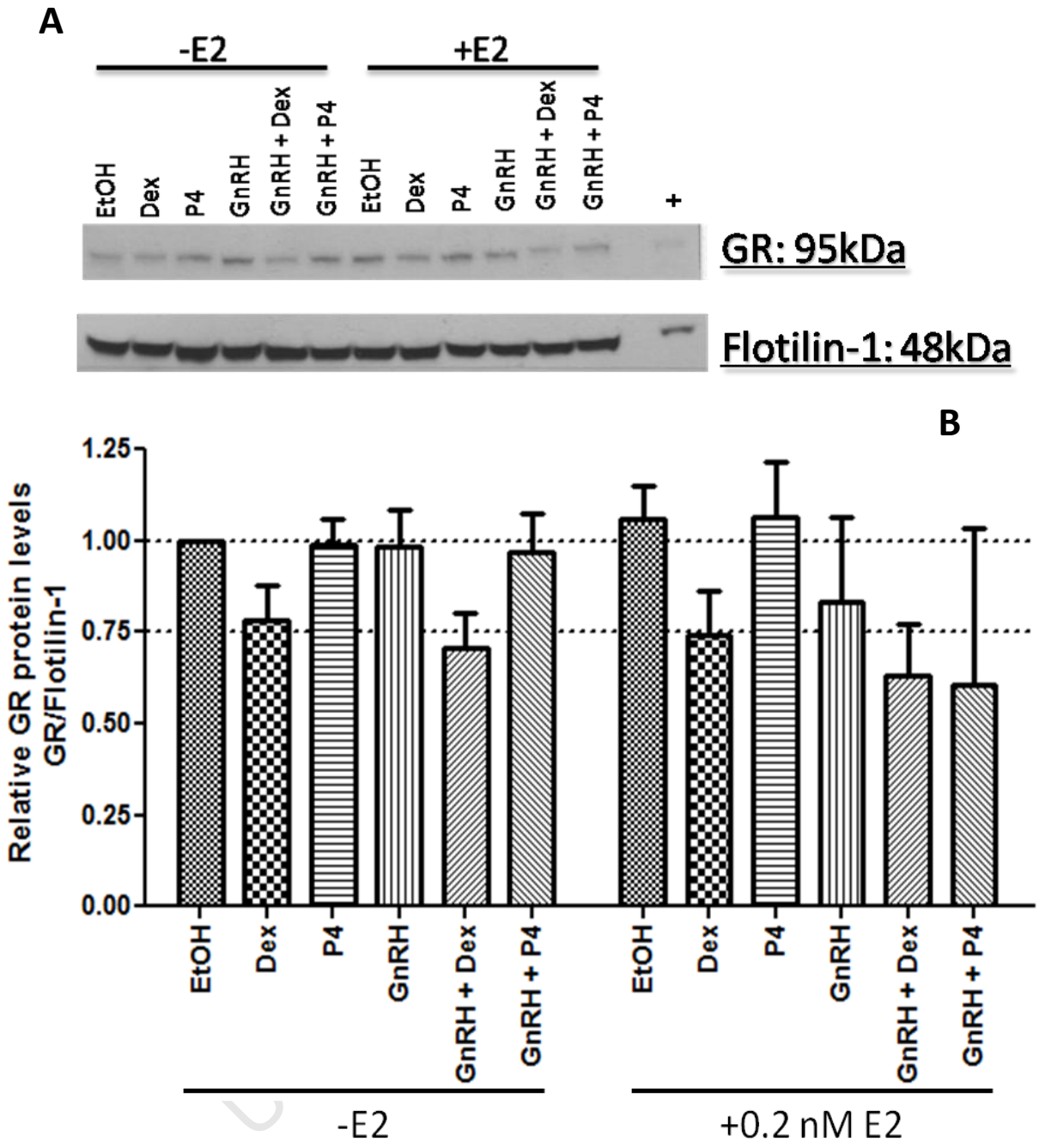


Figure 6.5 (A-B): Effects of Dex & GnRH, but not P4 and E2, on GR protein levels correlate with GR mRNA levels. LβT2 cells were plated into 12-well plates in DMEM supplemented with 10% serum. After a 24 hour incubation period, the cells were treated with or without 0.2 nM E2 in phenol red-free media supplemented with 10 % charcoal stripped serum. After 48 hours, the cells were treated with 100 nM of the appropriate ligand and combination thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting in 50 µl SDS sample application buffer. Equal volumes (10 µl) of LβT2 whole cell lysates were separated on a 8% SDS denaturing polyacrylamide gel transferred to a nitrocellulose membrane and probed with anti-GRα-specific and anti-flotilin-1-specific (Loading control) primary polyclonal antibodies. Anti-rabbit secondary

antibodies were used for detection of GRα and anti-mouse-HRP secondary antibodies were used for detection of flotilin-1 primary antibodies. Signals were visualised using Amersham Chemiluminescence. GRα and flotilin-1 proteins are observed at 95 kDa and 48 kDa (A). GRα signals were developed on radioautographs and scanned. Signal intensities were quantified using AlphaEase FC™ Software (Alpha Innotech Corperation). Loading was normalised through flotilin-1 protein levels and represented as relative GR protein, relative to vehicle treated EtOH. The blot in A is a single representative of a GRα immune-blot, while the graph shows pooled results for several independent western blots (n=4 for all samples without E2, n=3 for all E2 containing samples, and n=1 for +E2 P4 sample).

Figure 6.5A is a representation of an immuno-blot showing varying GRα protein levels in response to multiple ligand conditions. No significant differences in total GR levels are seen (Figure 6.5B), unlike the results obtained for GR mRNA levels (Figure 6.4). The responses observed on relative GRα protein levels appear small and insignificant. Error in quantification and normalisation may have resulted in no significant changes GRα protein expression. The rate of GR turn-over in response to hormone treatments, may account for this apparent discrepancy in GRα protein levels (Avenant *et al.,* 2010).

Treatment with Dex appeared to reduce GR protein levels to 75% relative to vehicle, while P4 and GnRH treatments alone show no effect on regulating GR protein levels (Figure 6.5B). Dex and GnRH co-treatment appears to reduce GRα protein levels (70% relative to vehicle) (Figure 6.5B). This result corresponds to the suppressive combinational effects of Dex and GnRH co-treatment on *GRα* mRNA levels (Figure 6.4).

An apparent repressive trend is seen when comparing the effects of Dex and Dex plus GnRH conditions on total GRα protein levels (Figure 6.5B) with *GRα* mRNA levels (Figure 6.4). Not all ligand-dependent trends correlated with the GRα mRNA expression, as no effects were seen on GRα protein levels with P4 treatment, and no abolishment of Dex-mediated repression in response to E2 priming.

The effects 0.2 nM E2 on GRα protein levels yielded a range of insignificant responses which appear to be highly variable when compared to the un-primed sample set (Figure 6.5B). This may be a result of biological and technical variation between experimental repeat experiments. E2 priming alone showed no effect on GRα protein levels, while E2 priming and Dex treatment appeared to reduce GRα protein levels to 70% relative to vehicle (Figure 6.5B). P4 treatment on E2 primed LβT2 cells result in no effect on GR protein levels, with GRα levels remaining unchanged compared to vehicle (Figure 6.5B). GnRH treatments on E2 primed LβT2 cells appeared to show a reduction in GRα protein levels to about 80% relative to vehicle.

Co-treatments with Dex and GnRH on E2 primed LβT2 cells appear to show the largest reduction in GRα protein levels (about 60% relative to vehicle) (Figure 6.5B), in correlation with GRα mRNA expression levels (Figure 6.4). Finally the effects of P4 and GnRH co-treatments appear to show a highly varied response, with GRα protein levels dropping to 60% relative to vehicle (Figure 6.5B).

Taken together, E2 priming appears to increase the variability of GRα protein levels in response to ligand treatment (Figure 6.5B). This suggests that the quantitative analysis of GR protein through western blotting and subsequent computer scanning is not the most sensitive technique. This may explain discrepancies observed between GR mRNA and protein levels in response to ligand treatments. However, the trend in varying GR protein levels in relation to ligand treatments (Figure 6.5B) appears to correlate with the observed trend in *GRα* mRNA expression with regards to Dex and Dex plus GnRH treatments (Figure 6.4). Dex treatments suppress GRα mRNA and protein levels, in a ligand-dependent manner, with the combined effects of Dex plus GnRH being seen on Dex plus GnRH treatments, highlighting potential GR and GnRHR crosstalk regulating GR protein levels.

Therefore the down-regulation of GRα mRNA and protein levels in response to Dex and GnRH treatments may serve as feedback signalling mechanisms, to regulate GR-mediated signalling (Kotitschke *et al.,* 2009) To further confirm this, additional data sets are needed to be investigated using a more sensitive and accurate method of quantifying GRα protein levels (such as using digital imaging software opposed to using x-ray film for image development). A time course should also be implemented to measure the effects of Dex and

GnRH signalling on GnRHR expression relative to functional GR protein levels in LβT2 cells. This feedback signalling may have influential downstream effects of GR-mediated transcription in LβT2 cells.

CHAPTER 7

CONCLUSIONS

A central aim of this study was to assess the effect of GnRHR and SR signalling on endogenous hormone receptor mRNA levels in the LβT2 cell line. Receptors of interest included the GnRHR, and the ER, PR and GR steroid receptors. Basal steroid receptor expression and activity were characterised. The effect of hormone treatments on relative GnRHR and steroid receptor mRNA expression was examined using quantitative real-time PCR. Results presented in this study show significant ligand-dependent regulation of endogenous LβT2 GnRHR and steroid receptor mRNA expression. These results do not establish a precise mechanism of repression. But, they do establish a basis for further experimental assessment on the specific responses observed.

7.1 ER, PR and GR characterisation in the LβT2 cell line

Table 7.1: Characterisation of steroid receptors in the LβT2 cell line. Table summarizes results on basal mRNA expression, basal protein expression and agonist-mediated activity of target steroid receptors (ER, PR and GR) in the LβT2 cell line.

Steroid Receptor	Observed Results	Relevant Figures
ERα	ERα mRNA is detected with conventional PCR	**Figure 4.1**
	No ERα protein detected with Western blotting	**Figure 4.2**
	No ER-mediated activity on ERE-luc reporter	**Figure 4.3**
PR-B	PR-B mRNA is detected with conventional PCR	**Figure 5.1**
	No PR-B protein detected with Western blotting	**Figures 5.2 & 5.4**
	No PR-B-mediated activity on PRE-luc reporter	**Figure 5.3**
GRα	GRα mRNA is detected with conventional PCR	**Figure 6.1**
	GRα protein detected with Western blotting	**Figures 6.2 & 6.4**
	GR-mediated activity on GRE-luc reporter	**Figure 6.3**

In this study it was shown that ERα mRNA is basally expressed in the LβT2 cell line (Figure 4.1), but assessment of ERα protein levels proved inconclusive. The ERα-specific primary antibody used appeared to be unsuccessful in detecting of ERα protein (Figure 4.2), as was shown from antibody optimization for the ERα primary antibody (data not shown). Furthermore ERE-luc reporter promoter assays showed no change in ER-mediated activity upon agonist (E2) treatment (Figure 4.3). The same ERE-luc reporter plasmid did respond to

GnRH treatments (Figure 3.5), suggesting a response independent of the ER. ER isoforms were not assessed in this study, leaving the possibility that a truncated or even mutated ER isoform may be present in the LβT2 cell line. A previous study has shown the presence of functionally active ERα protein in the LβT2 cell line (Shupnik *et al.,* 2000; Chen *et al.,* 2009), yet the current study was unsuccessful in detecting any ER protein and activity. Therefore in conclusion, ERα mRNA is basally expressed in the LβT2 cell line (Figure 4.1), and is ligand-dependently regulated in response to hormone treatments (Figure 3.4). This suggests the ERα is likely to have some functional purpose in LβT2 responses (Chen *et al.,* 2009).

This study has shown that PR-B mRNA is basally expressed in the LβT2 cell line (Figure 5.1). However, no PR-B protein could be detected using Western blotting techniques (Figures 5.2 and 5.4). This result was further confirmed, as PR (A+B) primary antibody was shown to be specific for the PR protein during PR (A+B) antibody optimization in the LβT2 and Cos-7 cell lines, however this data is not shown. The results of the reporter assay show no significant levels of agonist (P4 and R5020) activated PR-B protein (Figures 5.3 and 5.6). Interestingly, P4 treatments did significantly affect ERα mRNA expression (Figure 4.4) and GRα mRNA expression (Figure 6.4), suggesting the presence of a progesterone receptor mediating the transcriptional effects of P4 on target gene-expression in the LβT2 cell line.

One weakness of this study was in the failure to detect ERα and PR-(A+B) protein, despite the detection of their respective RNAs by conventional and real time PCR. Interestingly, new studies are beginning to reveal a complex relationship between SRs and endogenous miRNAs. miRNAs are an abundant class of small nonprotein-coding RNAs that mostly function as negative regulators of protein-coding gene expression in multiple tumor cell lines (Cochrane *et al.,*2010). Several miRNAs have been shown to directly target and repress the translation of ERα. These include miR-206, miR-222, miR-22, miR-18a, miR-19b, miR-20b and miR145 (Cochrane *et al*., 2010; Mallot *et al*., 2009; Kondo *et al*., 2008; Xiong *et al*., 2010; Zhao *et al.,* 2010). Overexpression of these miRNAs has been shown to decrease ERα protein and suppress ERα-mediated signalling (Cochrane *et al*., 2010). Futhermore, two miRNAs, the production of which was inhibited by estrogen, have been shown to target PR and reduce PR mRNA and protein levels (Maillot *et al.,* 2009). Whether this relationship between SR expression and miRNAs is responsible for the lack of ERα and PR-B protein

detection in the LβT2 cell line still needs to be determined. This could be assessed through the use of real time PCR, using LβT2 RNA samples and stem-loop RT primers specific for the miRNAs in question (Vreugdenhil et al., 2009; Maillot et al., 2009). With regards to the GR, it was shown that GR protein is present and functionally active in the LβT2 cell line. Results show basal GRα mRNA expression (Figure 6.1) and basal GRα protein expression (Figure 6.2). Results further show functional GR-mediated transcription in response to GR agonist (Dex) treatment on a GRE-reporter plasmid (Figure 6.3), consistent with the literature (McGillivray *et al.,* 2007; Kotitschke *et al.,* 2009). Furthermore GR protein levels appear to be regulated in response to ligand treatments, suggesting that the GR is likely to have a functional purpose in mediating LβT2 responses (Figure 6.4). Taken together, these results show functionally active GRα in the LβT2 cell line.

7.2 Summary of the effects hormones have on SR mRNA expression

Table 7.2: Summary of mRNA expression results. In this study a number of ligand-dependent responses were seen affecting hormone receptor mRNA expression in LβT2 cells. The main results obtained from this study have been summarized in the table below.

Target of Interest	Observed Results	Relevant Figures
GnRHR mRNA levels	Appears to increase in response to Dex Appears to increase in response to GnRH Significant increase in response to Dex + GnRH E2 priming appears to increase the Dex response E2 priming appears to eliminate Dex additive effect	**Figure 3.2**
ER mRNA levels	Significant decrease in response to GnRH Significant decrease in response to P4 E2 priming appears to increase Dex response E2 priming appears to reduce Dex repressive response	**Figure 4.4**
PR mRNA levels	Significant increase in response to GnRH No effect in response to Dex, P4 and E2 treatment alone E2 priming appears to have no effect on PR mRNA expression	**Figure 5.4**
GR mRNA levels	Significant decrease in response to Dex Significant decrease in response to P4 Significant decrease in response to GnRH Significant decrease in response to Dex + GnRH E2 appears to inhibit Dex response E2 priming appears to reduce Dex repressive response	**Figure 6.4**

A success of this study revealed that the GnRHR gene and SRs genes (ER, PR and GR) are significantly regulated in the LβT2 cell line, showing reproducible ligand-dependent trends in response to hormone treatments. This suggests that promoter regions of these target genes are transcriptionally regulated via hormone-responsive *cis*-elements. The GnRHR-gene promoter has been extensively characterised (Sadie, 2006), showing multiple factors recognizing specific *cis*-elements within the GnRHR promoter (Figure 1.8). SR-gene promoters have been sequenced (Breslin *et al.,* 2001; Kos *et al.,* 2001; Kastner *et al.,* 1990), and show multiple promoters and transcriptional start sites. SR-gene promoters contain GC-rich regions and generally lack TATA or CAAT boxes (Breslin *et al.,* 2001; Kastner *et al.,* 1990), making interpreting mechanisms for transcrpitional regulation complicated. The author feels there is a lack of studies addressing the characterisation of *cis*-elements and molecular mechanisms mediating ligand-dependent SR-gene promoter regulation. For this reason, more research is required in this field.

Interestingly P4 treatments were seen to significantly down-regulate ERα (Figure 4.4) and GRα mRNA expression (Figure 6.4). How these effects of P4 are being mediated to regulate target LβT2 gene expression is unclear, given the lack of detectable functional PR protein. P4 has been shown to be a partial agonist for the GR, having a relative binding affinity (RBA) of 274 nM for P4 binding to the GR; established in COS-1 cells by competitive binding assays using radio-labelled GR agonist, Dex, and transiently expressed recombinant GR protein (Ronacher *et al.,* 2009). The RBA for Dex binding to the GR was also calculated to be 14 nM (Ronacher *et al.,* 2009). Therefore one can estimate that the concentrations of P4 used in this study (100 nM) will not be high enough to saturate the GR, yet may have some partial effect on GR activity (Kontula *et al.,* 1983; Koubovec *et al.,* 2005).

However a role for the GR in mediating the effects of P4 is not supported by the results showing significant differences between Dex and P4 treatments on ERα mRNA expression (Figure 4.4). ERα mRNA expression is significantly down-regulated in response to P4 treatment, yet is not regulated in response to Dex treatment (Figure 4.4). Therefore GR signalling appears unlikely to be responsible for the effects of P4, suggesting a specific P4-signalling pathway.

Another possibile mechanism by which P4 may elicit these responses is thought to be via a putative mPR. However, the results in this study suggest that mouse mPRα mRNA is not expressed in the LβT2 cell line (Figure 5.7). The presence of mPRα protein could not be determined due to problems in recombinant mPRα protein expression and mPRα protein detection using Western techniques (Addendum A, Table 9.1). The effects of P4 may be mediated by mPRβ or mPRγ, the expression of which was not examined in the current LβT2 study. However, this is unlikely as others have shown that mPRβ and mPRγ mRNA are not expressed in the pituitary of humans (Zhu *et al.,* 2003).

A number of studies assessing the role of PR have been performed in the LβT2 cell line and have shown the presence of transcriptionally active PR-B protein (Thackray *et al.,* 2009; An *et al.,* 2009; Sleiter *et al.,* 2009). However, these studies also used transiently transfected PR protein to confirm a role for PR signalling. The current author queries the need to over-express PR protein, and the physiological significance of doing PR studies in a cell line which appears to exhibit little to no functional PR protein (Figure 5.1), when others have shown endogenous PR in both primary rat and mice models (Turgeon and Waring, 2000; Turgeon and Waring, 2006). This further highlights the importance of doing research in primary cell lines, as it may be possible for immortalised cell lines to change their properties over time under laboratory conditions. Taken together, P4 treatments did elicit a significant response on both ER and GR mRNA expression (Figures 4.4 and 6.4), supporting the concept that a functional PR protein is most likely present at low levels in the current LβT2 cell line.

Interestingly GnRH treatments were seen to affect the transcriptional expression of all steroid receptors examined in this study (Figures 4.4, 5.4 and 6.4), highlighting the importance of GnRH signalling feedback in regulating gonadotrope steroid receptor levels. To the knowledge of the author, these are the first studies showing the effects of GnRH on endogenous SR mRNA expression in LβT2 cells.

Mechanisms explaining how GnRH is able to regulate target receptor expression are still unknown, and require further experimental assessment. Two possible mechanisms could account for the observed regulation of target steroid receptor gene expression: The first may be via GnRH activating a wide variety of signalling proteins indirectly involved in gene transcription (PKA, PKC, MAPK) (Marinissen and Gutkind. 2001). These kinases may then

target transcription factors (including c-Jun and c-Fos) to regulate *cis*-elements in target SR promoter regions. A site resembling an NF-κB in the GRα 1C promoter region (Figure 1.14) (Breslin *et al.,* 2001), and an AP-1 site found in the ERα promoter (Tang *et al.,* 1997) may be potential promoter targets for transcriptional regulation in response to GnRHR signalling (Kotitsche *et al.,* 2009; von Boetticher, 2008; Marinissen and Gutkind, 2001).

The effect of GnRH in modulating SR activity through ligand-independent mechanisms has been reported in a number of published studies. GnRH has been shown to ligand-independently activate the GR (Kotitschke *et al.,* 2009), ER (Chen *et al.,* 2009) and PR (An *et al.,* 2009) in LβT2 cell line. These studies show ligand-independent phosphorylation of SRs at key amino acid residues in response to GnRH treatment. Therefore a second mechanism may be via GnRHR activating SRs, in the absence of steroids, to regulate promoter activity. Potential targets for SR-mediated regulation include a half-GRE site in the GRα 1C promoter region (Berslin *et al.,* 2001), a half-ERE in the PR-A promoter (Kastner *et al.,* 1990) and, a half-PRE site in the ERα promoter (Amicis *et al.,* 2009).

One final explanation could involve a combination of GnRH-induced ligand-independent SR activation, and conventional GnRH-mediated signalling. Although one can hypothesise a mechanism based on the current literature, further experimental information on the ligand-dependent regulation of endogenous SR promoter expression would help in assessing the observed transcriptional effects GnRH has on target SR genes

Combination treatments of hormones were seen to significantly modulate mRNA receptor expression in LβT2 cells. Specifically, Dex and GnRH co-treatments were seen to act additively too significantly up-regulate GnRHR mRNA expression. As explained earlier, this result confirms a mechanism previously suggested (Kotitschke *et al.,* 2009). Dex plus GnRH treatments also significantly down-regulate GRα mRNA expression more so when compare to individual treatments alone (Figure 6.4). Dex-mediated ligand-dependent activation of the GR, and GnRH-mediated ligand-independent activation of the GR, may mediate the transcriptional effects seen the GRα mRNA expression. Therefore two separate signalling pathways may be activated in response to Dex and GnRH alone, yet both pathways may converge on the GRα promoter to potentiate the observed Dex + GnRH repression on GRα mRNA expression. Furthermore the GRα 1C promoter contains a half-GRE site and an NF-κB

site (Figure 1.14) (Breslin *et al.,* 2001), two potential *cis*-elements that may be involved in this response. However, this hypothesis requires further experimental assessment before any conclusions can be made.

An interesting trend was seen with E2 priming appearing to modulate Dex responses regulating GnRHR (Figure 3.2), ER (Figure 4.4) and GR (Figure 6.4) mRNA expression levels. This response was not apparent for all genes (Figure 5.4), suggesting it is gene-specific. In the literature, the effect of 0.2 nM E2 priming has been examined in the context of PR-mediated signalling, as E2 priming shows a positive influence on PR expression in primary rat and mice gonadotropes (Turgeon and Waring, 2006; Turgeon and Waring, 2000). In this current study, it appears that E2 may have a broader effect on SR-mediated transcription. Low dosages were seen influencing the transcriptional effect Dex treatments may have on endogenous SR mRNA levels in the LβT2 cell line. These results suggest that E2 and Dex signalling pathways may crosstalk along the HPG and HPA axes (Figure 1.4). Yet molecular mechanisms of crosstalk between estrogens and glucocorticoids are poorly understood. Interestingly two studies have shown E2 treatment can inhibit glucocorticoid action. The first study shows that ligand-bound ERα can block Dex-mediated repression on the IL-8 promoter in U2Os cells (Cvoro *et al.,* 2011). Here, ERα directly interacts with the GR to interfere with the recruitment of nuclear co-activator 2 to the IL-8 promoter (Cvoro *et al.,* 2011). A second study shows that E2 can indirectly decrease ligand-induced GR phosphorylation at Ser-211 in MCF-7 and T47D cells (Zhang *et al.,* 2009). Here, E2 increased the expression of protein phosphatase 5 (PP5), a phosphatase involved in dephosphorylating the GR at Ser-211, to decrease the transcriptional activity of GR protein (Zhang *et al.,* 2009). The question whether this effect of E2 priming is mediated directly by ER and GR interactions or indirectly through regulating PP5 expression requires further experimental assessment.

7.3 The effects of E2 and GnRH priming on reporter promoter activity

Reporter Promoter	Observed Results	Relevant Figures
ERE-reporter promoter	Agonist treatments show no increase in ERE-transcription with and without GnRH priming GnRH priming significantly increases basal ERE-reporter activity	**Figure 4.5**
PRE-reporter promoter	Agonist treatments show no increase in PRE-transcription with and without GnRH priming GnRH priming significantly effects basal PRE-reporter activity	**Figure 5.6**
PRE-reporter promoter	Agonist treatments show no increase in PRE-transcription with and without E2 priming E2 priming significantly effects basal PRE-reporter activity	**Figure 5.6**

The aim of the reporter-promoter assays was to assess if E2 or GnRH priming could modulate SR (ER and PR) activity on a HRE-reporter plasmid, in the absence and presence of agonist. ER-specific agonist treatment (E2) and PR-specific agonist (R5020) showed no significant increase in ERE-mediated and PRE-mediated transcription, respectively. Both results have been discussed previously in Section 7.1.

Interestingly, when LβT2 cells were primed with GnRH, a significant increase in basal ERE and PRE-reporter activity was observed. Previous studies have highlighted the ligand-independent effects of GnRH on the ER and the PR in the LβT2 cell line (Chen *et al.,* 2009; An *et al.,* 2009). These studies show that ER and PR activation is mediated by GnRH treatments, inducing site specific phosphorylation and co-activator interaction to promote endogenous fosB and fshβ mRNA expression, respectively, and ERE-or PRE-reporter promoter activity in LβT2 cells (Chen *et al.,* 2009; An *et al.,* 2009). Unfortunately the hypothesised GnRH-mediated ligand-independent SR activation cannot be confirmed from HRE-luc reporter results alone. Further experiments are required before any conclusions may be made. A more plausible conclusion may suggests GnRH-priming is modulating the activity of RNA polymerase II or basal transcription factors involved in HRE-reporter expression, as no functional ER or PR protein could be detected in the current LβT2 cell line (Figures 4.2, 4.3, 5.2, 5.4).

Furthermore, a significant increase in PRE-reporter activity was seen in response to 0.2 nM E2 priming. The RBA of the ERα for E2 has been experimentally determined to be 0.9 nM

(Blair *et al.,* 2000). Therefore at a concentration of 0.2 nM E2, only a small fraction of the ER would be occupied by E2. This suggests the increase in PRE-reporter activity (in response to E2 priming) may not be mediated by liganded ER. However, one can only conclude that further experimental data is required to investigate the potential involvement of ER protein in this significant response.

7.4 Statistical analysis of SR mRNA expression data

An important aspect of all biological research is the statistical significance of the data collected during any study in question. In order to gather statistically relevant data, the researcher must be unbiased in interpreting his/her results, and appropriate statistical analysis should be applied. Therefore quantitative real-time PCR expression data was analyzed using a non-parametric two way ANOVA with bonferroni post tests. This ANOVA test was chosen to assess the significance of hormone treatments (variable 1) and E2 priming (variable 2) on endogenous LβT2 GnRHR and SR mRNA levels. Furthermore, bonferroni post test were used to assess the significance of individual ligand treatments relative to control (-E2 EtOH).

A limitation of this statistical test occurs when using large number of hormone conditions (large sample set). A central aim of this thesis was the broad assessment of hormone-dependent effects on multiple genes in the LβT2 cell line, with the intent to establish a basis for further research. A large number of biological and technical repeats are required to obtain statistical significance for small differences on a large sample set. In this study, four biological repeats were collected for the un-primed (–E2) sample set, and three biological repeats for the E2 primed (+E2) sample set. Ideally more experimental repeats would be preferred for a study of this nature. However due to financial and time constraints, repeat number (n) was kept to a minimum to allow for the high number of hormone conditions. This may imply that some of the smaller insignificant responses observed on target mRNA levels may hold more significance if additional repeat experiments are performed.

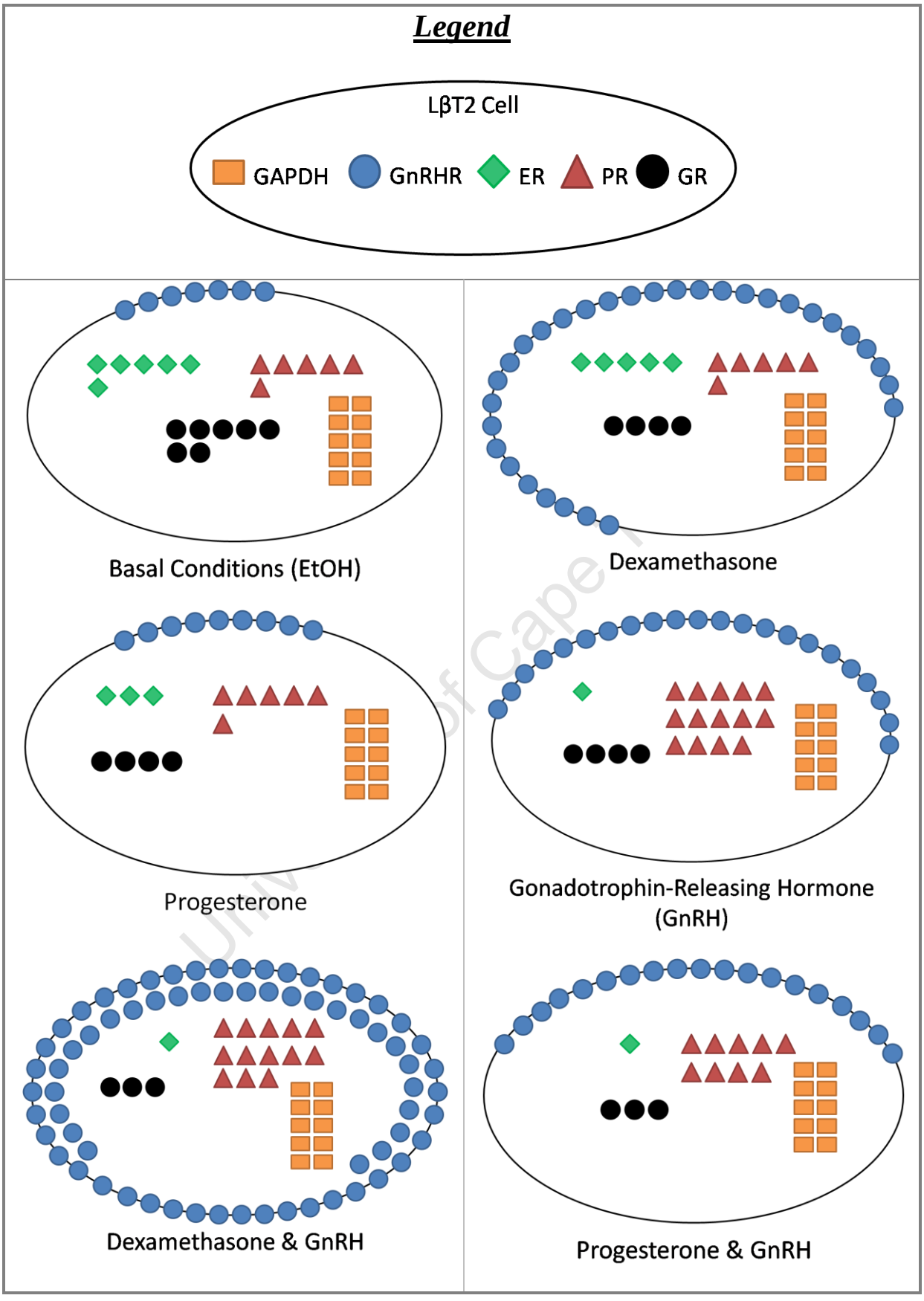
For this reason effects of hormone treatments on endogenous LβT2 *GnRHR* mRNA expression did not reveal much statistical significance in responses. However, this statistical insignificance is not surprising, as is most likely due to the large sample set (12 hormone

conditions) and low number of biological repeats (n=3), coupled to possibly small fold changes. The large degree of variability for the two ligand conditions (-E2 and +E2, Dex + GnRH treatments), further reduces any possibility of getting statistically significant data. What can be said is that there is a reproducible ligand-dependent trend regulating *GnRHR* mRNA expression in the LβT2 cell line (Figure 3.2), which appears similar to the results previously seen (Kotitsche *et al.,* 2009; von Boetticher, 2008). Kotitsche *et al,* (2009) showed that *GnRHR* mRNA expression is synergistically up-regulated in response to Dex plus GnRHR co-treatment. It was shown that the transcriptional effects of Dex and GnRH treatment are mediated via an AP-1 site on the GnRHR promoter, through a mechanism dependent on the GR and the GnRHR (Figure 1.8) (Kotitschke *et al.,* 2009). These results further show the potential for HPA and the HPG crosstalk regulating LβT2 gene expression. Taken together, the current study supports the finding that Dex, GnRH and co-treatment with both ligands, up-regulate *GnRHR* mRNA expression (Kotitsche *et al.,* 2009).

In an attempt to reduce the error present in real-time PCR data, one could incorporate a more accurate normalisation method of real-time PCR data by geometric averaging of multiple internal control genes (Vandesompele *et al*., 2002). These authors showed that the use of a single gene for normalisation may lead to a relatively large error in a significant proportion of the samples tested. This normalisation stratagy has been validated, and presents the possiblilty of studying the biological relevance of small expression differences (Vandesompele *et al.,* 2002).

7.5 A model to represent receptor mRNA levels relative to endogenous GAPDH mRNA expression

It is becoming clearer that a cell system exists in a constant state of flux, with multiple signalling events occurring constantly to maintain cellular homeostasis. This suggests that the expression of multiple target genes is regulated simultaneously in response to multiple signalling molecules. In the context of this study, varying SR-levels will have a significant consequence on down-stream SR-mediated signalling and/or target gene expression. Graphically representing total mRNA expression data relative to GAPDH mRNA expression will help visualise the "bigger picture"; the effects hormone treatments have on multiple receptor mRNA levels in the LβT2 cell line. Therefore, a model that integrated all the relative levels of target mRNA expression, was devised to visualise ligand-dependent trends on LβT2 target gene expression (Figure 7.1). The model assumes that the transcriptional effects of hormone treatments on SR mRNA levels have a concomitant effect on protein translation. However this is not necessarily the case (Malys and McCarthy, 2011), but is a useful assumption for estimating the relative receptor levels of LβT2 cells under different hormone conditions.


Legend
LβT2 Cell
GAPDH
GnRHR
ER
PR
GR
Basal Conditions (EtOH)
Dexamethasone
Progesterone
Gonadotrophin-Releasing Hormone (GnRH)
Dexamethasone & GnRH
Progesterone & GnRH

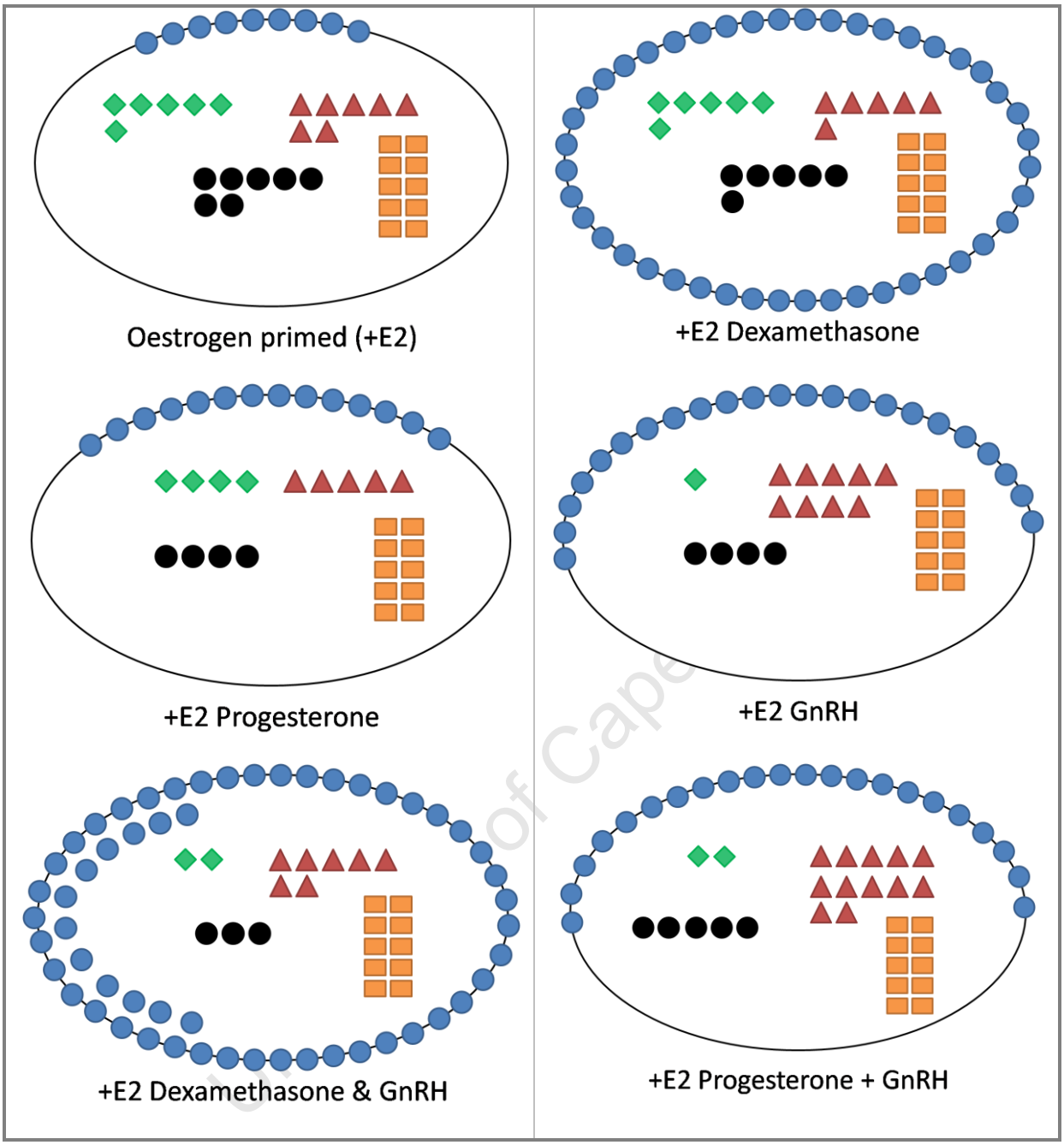


Figure 7.1: A cellular representation of total receptor levels in response to treatments, assuming relative protein levels follow relative mRNA levels. Using quantitative real time PCR data, Ct values obtained under basal (EtOH) conditions were averaged (n=3) for each gene of interest (GAPDH; GnRHR; ER; PR; GR). Total receptor levels were normalised to GAPDH expression under basal conditions (EtOH). Ligand-dependent effects were set relative to (EtOH) by applying the receptor ratio under basal conditions to the ligand-depend trends (ΔCt) observed for each receptor and each condition. Note, this model only represents a projection of relative cellular receptor content from quantitative real-time PCR, with the possibly incorrect assumption that the transcriptional effects on

receptor mRNA levels is translated through to protein levels. This model serves as a visualising aid, to help gain insight into the overall effects hormone treatments have on total receptor expression.

Firstly, an aspect of this study that has fallen short was in the assessment of SR protein levels. GRα protein levels were detected and appear to be regulated in a similar ligand-dependent trend as GRα mRNA, in response to Dex and GnRH treatments. The question whether ERα, PR-B and GnRHR protein levels will follow similar ligand-dependent trends cannot be concluded, as ERα and PR-B protein could not be detected using Western blot techniques. Furthermore, previous attempts in detecting GnRHR protein levels, in the Hapgood lab, have proven unsuccessful due to problems in the immuno-detection of GnRHR protein (data not shown). What this model can offer is a possible prediction of ERα, PR-B and GnRHR levels under hormone conditions used in this study.

What is interesting is the interplay of these hormones in regulating multiple receptor mRNA levels in the LβT2 cell line. Therefore this model helps to define a complete receptor transcriptome for the LβT2 and for the effects of these hormones, both alone and in combination.. A recent review has shown a role for multiple and simultaneous hormone treatments regulating gonadotrope physiology (Thackray *et al.,* 2010). This review suggests that a precise interplay of GnRH, activin and sex hormones is influential in governing gonadotropin hormone production. This also highlights the importance of hormones acting additively and even synergistically in regulating target gonadotrope genes (Thackray *et al.,* 2010). Whether these fold changes in receptor levels seen in this study, in response to multiple hormone treatments, is enough to result in a physiological effect, is hard to say. However, a study has shown that varying GR protein levels do have an influential effect on Dex responsiveness (Zhao *et al.,* 2003). Therefore one can hypothesis that these changes in receptor levels are influential in LβT2 physiology. Further studies in primary cultures are required to assess the effects on gonadotrope physiology.

An initial observation is the extent to which the GnRHR is regulated. Under all conditions, the GnRHR mRNA is never down-regulated. This may suggest that the GnRHR is an important receptor for maintaining LβT2 function. Previous studies have shown GnRHR-mediated signalling regulates a number of important gonadotrope genes, including fshβ (An *et al.,* 2009) fosB (Chen *et al.,* 2009) and LHβ (Turgeon *et al.,* 1996). Furthermore, under

GnRH + Dex conditions the GnRHR mRNA is highly expressed, in excess of any other receptor mRNA. This response appears to be synergistic in nature, and further supports the possibility that signalling crosstalk may lead to differential mRNA expression (Thackray *et al.,* 2010). This in not unexpected, as the main function of the pituitary gonadotrope is to respond to GnRH, suggesting that GnRHR-mediated signalling will be favoured under the combined Dex and GnRH hormone condition.

Interestingly ERα and GRα mRNA and GRα protein were not up-regulated in response to the hormone conditions used in this study. This result suggests negative feedback from the gonads and adrenal gland to the pituitary regulates relative SR levels. This feedback has been established, with GRα levels down-regulated in response to GCs (Burnstein *et al.,* 1990) and ER levels down-regulated in response to P4 (Amicis *et al.,* 2009). However, what is interesting, is the significant affect GnRH treatment has on reducing both ERα and GRα expression levels.

On the other hand, PR levels do not seem to be affected by Dex P4 and E2 treatments. Only GnRH treatments up-regulate PR levels in the LβT2 cell line. As for the effects of E2 priming, it appears no significant changes are occurring on mRNA the mRNA levels investigated. The only apparent effect is under Dex conditions, where Dex-mediated suppression of GRα is lifted in response to E2 priming. Taken together, it is clear that GnRHR, ER, PR and GR expression levels are regulated in response to hormone treatments. However, the LβT2 cell line may not mimic the effects in primary cells, and therefore need to be confirmed in primary cell cultures.

In conclusion, the most important observation from this study shows the influential role of GnRHR-signalling in regulating LβT2 mRNA expression. Not only were most of the significant changes in SR mRNA levels in response to GnRH treatment, but GnRHR mRNA levels were shown to be highly regulated in response to multiple hormone treatments. These results show GnRH-signalling significantly regulates ER, PR and GR mRNA expression in LβT2 cells, a result which has not been previously published.

7.6 Final conclusion

In summary, this current study presents for the first time, an overview of classical SR (ER, PR and GR) presence and function in the LβT2 cell line The study shows that GnRHR and SR (ER, PR and GR) mRNA levels, and GR protein levels are ligand-dependently regulated in LβT2 cells. This suggests that target SR promoters are transcriptionally regulated in response to multiple hormone treatments. Specifically, several interesting and significant findings were established from this study.

The significant findings in the LβT2 cell line include;

- GnRH treatment significantly regulates ER, PR and GR mRNA levels
- GnRH and E2 priming significantly modifies basal HRE-reporter activity
- GnRHR mRNA levels are significantly up-regulated in response to Dex and GnRH
- ERα mRNA levels are significantly down-regulated in response to GnRH
- GRα mRNA levels are significantly down-regulated in response to Dex and GnRH
- E2 priming appears to dampen the Dex response on ERα and GRα mRNA expression.
- Combination treatments of GnRH and P4 show no additive effect on ligand-dependent responses.
- E2 priming alone has no effect on GnRHR, ERα, PR-(A+B) and GRα basal mRNA levels.
- E2 priming does not affect P4 and GnRH-mediated responses.

Although no mechanisms were established for the multiple observed responses, this study does serve as a basis for further research into novel HPG and HPA crosstalk mechanisms regulating endogenous GnRHR, ER, PR and GR mRNA and protein levels in the LβT2 cell line. It is more than likely that SRs are involved in mediating the regulation of some of these target genes (Figure 1.9), however many questions remain unanswered. Therefore additional experiments will be required to follow up the responses observed in order to further interpret and assess mechanisms mediating the observed ligand-induced effects on SR mRNA expression in the LβT2 cell line.

CHAPTER 8

FUTURE PERSPECTIVES

The results presented in this study show significant changes in GnRHR, ER, PR and GR mRNA expression levels in response to multiple hormonal treatments in the LβT2 gonadotrope cell line. Many questions remain unanswered, including the presence of functional ER and PR protein and the molecular mechanisms that mediate some of the observed responses. For this reason additional experimental strategies are required to determine possible explanations for the observed responses in this study.

8.1 Basal ER and PR protein expression in the LβT2 cell line

No functional ER protein was detected in the LβT2 cell line, contradictory to previous LβT2 studies (Chen *et al.,* 2009; Shupnik *et al.,* 2000). Although the primary ERα antibody used for western blotting proved unsuccessful in detecting ERα protein (Figure 4.2), another ER antibody can be ordered to re-assess ER protein levels in this cell line. An additional strategy to assess whether functional ER protein is present in the LβT2 cell line would be to reproduce the real-time PCR results shown by Chen *et al.,* (2009). If GnRH treatments up-regulate endogenous fosB mRNA expression, it would suggest that functional ER protein is present, as the literature reports that ERα protein mediates the transcriptional effects of GnRH on the fosB promoter in a ligand-independent manner (Chen *et al.,* 2009).

Previous studies have shown a functional role for PR-mediated signalling in the LβT2 cell line (An *et al.,* 2009; An *et al.,* 2006). No PR protein could be detected in the LβT2 cell study using western blotting techniques in this study, however, the functional PR protein reporter assay (Figure 5.3), appeared to show a small increase in PR-mediated transactivation upon agonist treatment. Only three replicates were performed, however. Therefore additional repeats of the PRE-luc reporter experiment are required to assess the question of whether LβT2 cells express low levels of functional PR protein in a statistically relevant manner. Interestingly significant changes in ER (Figure 4.4) and GR (Figure 6.4) mRNA expression are seen in response to P4 treatments, suggesting the presence of a functional progesterone

receptor. A different experimental strategy to address whether LβT2 cells have functional PR protein could involve reproducing the real-time PCR results presented by An *et al.,* (2009). If it is shown that GnRH treatments can significantly up-regulate endogenous fshβ mRNA expression, it would suggest functional PR protein is present in the LβT2 cell line. as the literature reports that the PR-B ligand-independently mediates the transcriptional effects of GnRH on endogenous fshβ mRNA expression (An *et al.,* 2009).

Another challange presented by this study was establishing which PR isoform is predominantly expressed in the LβT2 cell line. The primers used for amplifying PR mRNA expression were not suitable for distinguishing differences between PR-B and PR-A isoforms. Therefore PR-B and PR-A-specific primers should be used to investigate firstly if both isoforms are present, and secondly at what ratios of PR-B/PR-A mRNA levels exist in the LβT2 cell line.

8.2 Mechanisms for GnRH-mediated regulation of SR levels

Interestingly GnRH treatment was shown to significantly affect mRNA expression levels of the ER, PR and GR in the LβT2 cell line. This may occur via two possible mechanisms. The first may be via GnRHR signalling activating intracellular signalling cascades to activate various signalling complexes and transcription factors to initiate a transcriptional response. A site resembling an NF-κB in the GRα 1C promoter region (Figure 1.14) (Breslin *et al.,* 2001), and an AP-1 site found in the ERα promoter (Tang *et al.,* 1997) may be potential promoter targets for transcriptional regulation in response to GnRHR signalling. To confirm that the effects of GnRH treatments on target SR mRNA expression are mediated by the GnRHR signalling pathway; siRNA transfections can be performed to silence basal *GnRHR* mRNA expression and investigate GnRHR-mediated responses.

The second mechanism may be via GnRHR/SR crosstalk, whereby GnRH mediates SR activation via steroid-independent mechanisms (An,*et al.* 2009; Chen,*et al.* 2009; Kotitsche *et al.,* 2009). Therefore knock down studies using GR-, PR-or ER-specific siRNA tranfections can be used to silence GR, PR or ER mRNA expression and determine if the responses to GnRH are dependent on unliganded SRs. If SRs are shown to mediate transcriptional responses to GnRH treatments on some target genes, the question arises as to whether post-translational

modifications play an important role in these GnRH-dependent, but steroid-independent mechanisms. Previous studies have shown important amino acid residues involved in the GnRH-dependent activation of target SRs. Phosphorylation at Ser-118 and Ser-167 have been identified to coincide with ER steroid-independent, GnRH-dependent activation (Chen *et al.,* 2009). Phosphorylation at Ser-294 has been identified to coincide with PR steroid-independent GnRH-dependent activation (An *et al.,* 2009); and phosphorylation at Ser-234 has been identified to coincide with GR steroid-independent GnRH-dependent activation (Kotitschke *et al.,* 2009). The question whether these particular serine residues are phosphorylated in response to GnRH can be assessed with overexpressed mutant SRs which cannot be phosphorylated at target serine residues.

In both cases, insight into the transcription factors and *cis*-elements involved in mediating this transcriptional regulation of target SR genes would allow for a more thorough mechanistic understanding of the responses observed in this study. Therefore it would be interesting to utilize co-immuno precipitation (Co-IP) and chromatin immuno precipitation (ChIP) experiments to investigate which transcription factors are recruited to the target promoter, and the regions/*cis*-elements to which they are recruited.

Finally, significant effects were seen on basal HRE-luc reporter activity in response to GnRH priming. Both ERE-luc (Figure 4.5) and PRE-luc (Figure 5.6) expression increased in response to GnRH treatments. This may be a result of the ligand-independent activation of the ER or PR in response to GnRH. This mechanism is possible, since it has been shown that GnRH treatment mediates the site-specific phosphorylation of the ER and PR to increase endogenous LβT2 fosB and fshβ mRNA expression respectively (Chen *et al.,* 2009; An *et al.,* 2009). However, it is also possible that these responses are independent of SRs, and mediated by GnRH activating component(s) of basal transcription machinery present on the HRE-reporter construct. The use of a mutated HRE-luc reporter should discriminate between these possibilities.

8.3 Ligand-dependent responses on ERα mRNA expression

P4 treatment significantly reduced endogenous ERα mRNA expression (Figure 4.4) in the LβT2 cell line. This result may demonstrate a direct mechanism for the anti-estrogenic effects of P4, as the suppressive effects of P4 on ERα expression have been observed in multiple reproductive cell lines (Weigel and deConick, 1993). A half PRE site is present in the ERα promoter and is functional in MCF-7 breast cancer cells (Amics *et al.,* 2009). This suggests that the effects of P4 may be via the PR binding to this half site. However, since functional PR could not be detected in these cells, the question arises whether this response is mediated by the PR in the LβT2 cell line. To get a better understanding, Co-IP and ChIP experiments can be used to assess which transcription factors may mediate the observed response to P4, and with which *cis*-elements within the ER promoter protein complexes interact.

Another interesting question is whether P4 and GnRH co-treatment act in concert to repress ERα mRNA expression. Although a combination of these treatments was shown to repress ERα mRNA expression equal to that of GnRH treatment alone (Figure 4.4), it is possible with a subtracting concentration of GnRH. P4 co-treatment may act in concert with GnRH to suppress ERα mRNA expression. To assess this, an additional experiment can be performed using 100 nM, 10 nM and 1 nM GnRH treatments with and without 100 nM P4 treatments. This experiment would discriminate whether P4 has any additive properties with GnRH signalling.

Finally, the literature has shown that E2 is able to repress ERα expression in primary pituitary cells (Scheihofer *et al.,* 2000). However, in this current study, immortalised LβT2 cells appeared to show no change in *ERα* mRNA expression in response to E2 priming (Figure 4.4). This may be due to the low concentration (0.2 nM) of E2 used in the experiment. This difference in response to E2 between primary and immortalised pituitary cell lines shows a physiological difference between cell types. However to assess these differences, an additional experiment should be performed with LβT2 cells treated with a concentration of 10 nM E2 for 4 days (as performed by Scheihofer *et al.,* 2009), to assess if the LβT2 cells respond like primary gonadotrope cells, as stated in the literature.

8.4 Ligand-dependent responses on PR (A+B) mRNA expression

The current study showed little ligand-dependent variation on PR (A+B) mRNA levels. However it is possible that small significant changes do occur, which may be significant with additional repeat experiments. Designing more efficient PR primers may also improve the ability to detect small changes in PR mRNA levels, as the primer efficiency of the PR-A+B primer pair was calculated to be 75% (Addendum C, 11.3).

8.5 Ligand-dependent responses on GRα mRNA expression

It was shown that relative GR mRNA expression is significantly repressed (50%) in response to Dex treatments. An interesting question is whether the GR mediates this response in the LβT2 cell line. LβT2 cells transfected with GRα-specific siRNA to knock-down the GR will help evaluate the involvement of the GR. Additionally one could utilize a dominant-negative GR expression construct, lacking DNA binding and transcriptional capability, to allow further assessment of GR involvement in this response to Dex. It has been shown that multiple promoters exist in the human GR promoter, with glucocorticoids able to regulate endogenous GR gene expression (Breslin *et al.,* 2001). Furthermore a half-GRE site is present in the GRα 1C promoter region (Berslin *et al.,* 2001). This suggests the the effects of Dex may be via the GR binding to this half site. Therefore, it would be interesting to utilize Co-IP and ChIP experiments to investigate whether the GR is recruited to the GR promoter and the regions/*cis*-elements to which it is recruited.

In the case where Dex and GnRH treatments were seen to act in concert in repressing GRα mRNA (Figure 6.4), experiments are needed to assess whether this response is mediated by GR and GnRH crosstalk, or whether two separate signalling pathways are activated in response to Dex, and GnRH alone, yet converge on the GRα promoter to initiate the observed response. For this, GR and GnRHR knock-down studies may be performed using siRNA transfections. This would allow the researcher to assess the involvement of each receptor in mediating the response to Dex + GnRH treatments, and determine if GR and GnRHR crosstalk regulates GR mRNA expression.

Little information is available in the literature regarding the mechanisms of GRα-gene regulation in the LβT2 cell lines. It appears that most of the studies regarding the GRα-promoter region has been performed over a decade ago (Burnstein *et al.,* 1991; Strahle *et al.,* 1992), or performed in immune cell lines (Burnstein *et al.,* 1991; Ramdas *et al.,* 1999). Therefore, with the onset of new technologies including Co-IP and Chip experiments, a more detailed study on GRα-gene regulation could be performed.

8.6 E2 priming influencing Dex-mediated signalling

An interesting trend was seen with E2 priming appearing to modulate Dex responses in regulating GnRHR (Figure 3.2), ER (Figure 4.4) and GR (Figure 6.4) mRNA expression. These results suggest that E2 and Dex signalling pathways may crosstalk along the HPG and HPA axes (Figure 1.4). Yet molecular mechanisms of crosstalk between estrogens and glucocorticoids are poorly understood. Interestingly two studies have shown E2 treatment can inhibit glucocorticoid action. The first study shows that ligand-bound ERα can block Dex-mediated repression on the IL-8 promoter in U2Os cells (Cvoro *et al.,* 2011). Here, ERα directly interacts with the GR to interfere with the recruitment of nuclear co-activator 2 to the IL-8 promoter (Cvoro *et al.,* 2011). A second study shows that E2 can indirectly decrease ligand-induced GR phosphorylation at Ser-211 in MCF-7 and T47D cells (Zhang *et al.,* 2009). Here, E2 increases the expression of protein phosphatase 5 (PP5), a phosphatase involved in dephosphorylating the GR at Ser-211, to decrease the transcriptional activity of the GR protein (Zhang *et al.,* 2009). The question as to whether this effect of E2 priming is mediated directly by ER and GR interactions, or indirectly through regulating phosphatase expression can be determined using Co-IP experiments. If the ER is found to complex with the liganded GR in response to E2 priming, then it is an indication that the ER and GR directly crosstalk to regulate GRα mRNA expression. If the ER is not found to complex with the GRα, then it is possible that E2 priming may indirectly affect GR activity through phosphorylation, turnover, nuclear trafficking or co-factor recruitment. These subsequent questions can be answered using ChIP experiments to assess the effects of E2 priming on the GRα gene promoter.

8.7 Further statistical analysis for future experimental design

In order to further investigate interesting responses seen on SR mRNA levels, repeat experiments should be performed using only the hormone treatments of interest. This way more technical repeats could be collected with every biological repeat, thereby increasing the statistical significance of the data collected. Specific hormone responses of interest include:

- The effects of P4 of PRE-mediated transactivation
- The effects of Dex, GnRH and Dex + GnRH on GRα mRNA expression
- The effects of E2 priming in modulating Dex-mediated regulation of GnRHR, ERα and GRα mRNA levels

Before additional repeats experiments are to be performed, the researcher must focus on designing the experiment around which statistical analysis would be most appropriate for analysing the data set. For example, the researcher must take into account the number of variables of each experiment (for example, time, treatment or priming), in order to decide whether a one-way ANOVA or two-way ANOVA should be applied. Further statistical post-tests must be considered if the researcher is comparing experimental conditions to control (Dunette post-test) or comparing each experimental condition to each other experimental condition (Bonferroni post-test).

Finally, to further reduce experimental error present in a real-time PCR dataset, one could perform a more accurate normalisation method by geometric averaging of multiple internal control genes (Vandesompele *et al*., 2002), as mentioned in Section7.4.

8.8 Physiological relevance of LβT2 cell line studies.

An ongoing issue in the field of gene regulation is the relevance of results obtained in immortalised cell lines, as it may be possible that these cell lines do not respond in the same way as primary cells. On the other hand, immortalised cell lines greatly facilitate the investigation of cell-specific intracellular signalling. In the case of pituitary function, pituitary responses can be measured in primary pituitary cultures. However, these cultures typically

contain a mixture of cell types. For this reason, it is not always possible to determine whether any measured effects are direct or not, as these cultures have a heterogenic cell population that may be contributing to the final response measured. To resolve this, primary gonadotrope cells can be sorted from a pituitary cell population using flow cytometry. This is a technique that will hopefully be adapted by the Hapgood lab in assessing ligand-dependent effects on gene regulation in primary gonadotrope cells. This will be made possible through collaboration with Dr. Ulrich Boehm at the Institute for Neural Signal Transduction, Centre for Molecular Neurobiology, Germany (Wen *et al.,* 2010). On the other hand, primary cells cannot be maintained in continuous culture, creating practical constraints on experimental design. For this reason, this method can prove financially demanding and time consuming in gathering reproducible data.

However, performing experiments in immortalised cell lines can have its advantages over primary cell line work, as long as results are verified in a more physiological system, such as transgenic mice. Firstly, target cells can be immortalised at a particular developmental stage to represent different chronological differentiation stages of a cell line (Alarid *et al.,* 1996). Secondly, immortalised cell lines can be continuously maintained under laboratory conditions. For these reasons, immortalised mouse gonadotrope cell lines have been previously used to study mechanisms of gene regulation (Hapgood *et al.,* 2005). Yet, the author still feels it is in the best interest of all researchers to characterise immortalised cell lines used in experiments, and to compare cell-specific properties to primary cell types.

8.9 Improved experimental design for results presented in this current study

Only one repeat was obtained for the E2 primed P4 treatment for the real-time PCR data sets (Figures 3.2, 4.4, 5.4 and 6.4). The combined effects of E2 priming and 100 nM P4 treatment appeared to up-regulate GnRHR mRNA expression (Figure 3.2), and suppress both the ERα (Figure 4.4) and the GRα (Figure 6.4) mRNA expression, with no effects seen of PR expression levels. Statistical significance of these treatments where not established due to complications during sample collection and preparation. Therefore, additional repeats are required to clarify any statistical relevance of P4 treatments on E2 primed LβT2 cells.

Standards used to calculate GnRHR and GAPDH primer efficiencies were adopted from Kotitsche *et al.,* 2009, on the basis that identical protocols and cell lines were used between the two studies. However, it would be more appropriate to determine GnRHR and GAPDH primer efficiencies for each experiment in parallell, as primer efficiency can vary between each real-time PCR run. Under the assumption that additional data is required for publication, primer efficiencies should be determined in parallel with experimental sample sets, for each biological repeat. For this, a stock of pooled cDNA (1 or 2 dilutions) can be used to determine primer standards for each run, while sample cDNA is simutaniously assessed. Unfortunately due to time limits of the MSc, only one standard was performed for each primer pair (with the exception of GnRHR and GAPDH) in this study, Furthermore, it should be noted that the primer efficiencies for the ERα and GRα were calculated to be 113% and 130% respectively. High efficiency values indicate target mRNA transcripts are being amplified 2.13 and 2.3 times per PCR cycle for the ER and GR, respectively. Primer dimers may be one explaintion to the additional DNA amplication during real-time PCR cycles. Ideally, primer efficiencies between 80-100% are best for real-time PCR. Therefore to correct for inaccurate primer efficiencies, melting temperatures can be adjusted to correct for [E] values, or additional cDNA dilutions can be used during the standards to ensure accurate [E] value calculations. Finally, one could redesign primers to ensure more accurate [E] values. However due to time and finacial constrants, primer efficiencies for ERα and GRα were assumed as 2, suggesting 100% efficiency (which is still less efficient compared to calculated values). This assumption does not change the outcome of the relative expression data. If a [E] value of 130% was assumed for GRα expression, larger fold values would be seen, yet relative ligand dependent trends would remain unchanged. Although this approach is not the most ideal way to interpret expression data, it still served as a basis on which further experimental assessment can be performed.

This study showed the effects of agonist and GnRH treatments on endogenous ER- and PR-mediated HRE-luc reporter activity, but was not performed for GR transactivation due to time constraints. Additionally it would have been better to include a positive control (over-expressed SR) in reporter assay experiments to assess any problems that may have occurred during HRE-luc reporter plasmid transfection or ligand stimulation. For example, the results obtained in Figure 4.3 showing functional activity of endogenous ER protein, shows no

change compared to vehicle. This may be a result of problems experienced during transient transfection or ligand treatments. Therefore, a positive control for HRE-luc reporter activity using exogenous SR protein would assess whether transfection or ligand treatments were successful.

A technique that appeared to show relatively high variability between data sets was the quantification of western blots (Figure 6.5). The strategy of measuring band intensity of autoradiograms using scanning and AlphaEaseFC^{TM} imaging software was not the most reproducible, as many errors were introduced. These errors include the non-linear relationship between signal and intensity on the film, and the variation in background. For this reason, it is suggested that future western blot developments should use specialised imaging hardware (implementing digital camera technology) to increase reproducibility of western blot development, and ensure western blot exposures are always constant.

ADDENDUM A

SUPPLEMENTARY DATA

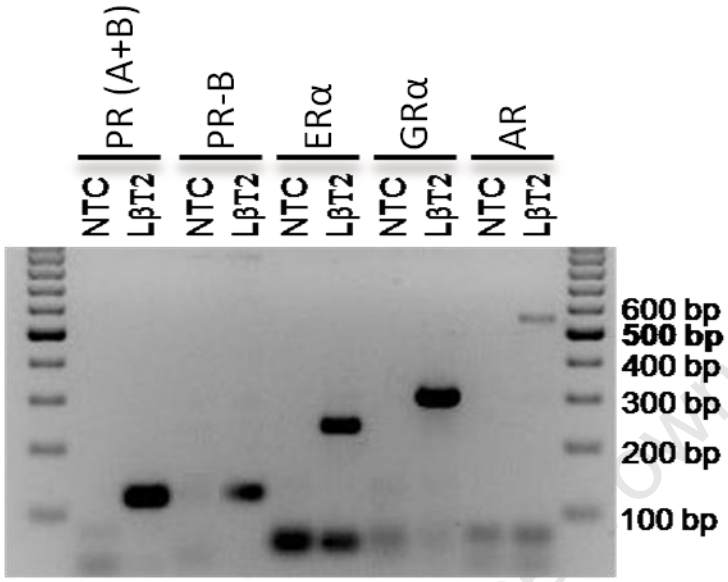


Figure 9.1: The LβT2 cell line expresses PR, ER, GR and AR mRNA under basal conditions. LβT2 cells were seeded into 6-well culture plates in DMEM supplemented with 10% FCS, and were left to grow to 70% confluency under basal conditions. Thereafter the cells were harvested and RNA extractions were performed, followed by a reverse transcriptase reaction to generate cDNA. Signals were amplified using conventional PCR with gene-specific primer pairs. PCR products were generated over 35 PCR cycles and separated on a 2% agarose gel by electrophoreses, and visualised with ethidium bromide staining. Positive signals are seen for PR (A+B) amplicon at 121bp; PR-B amplicon at 121 bp; ERα amplicon at 235 bp; GRα amplicon at 299 bp and AR amplicon at 544 bp. NTC defines the no template control.

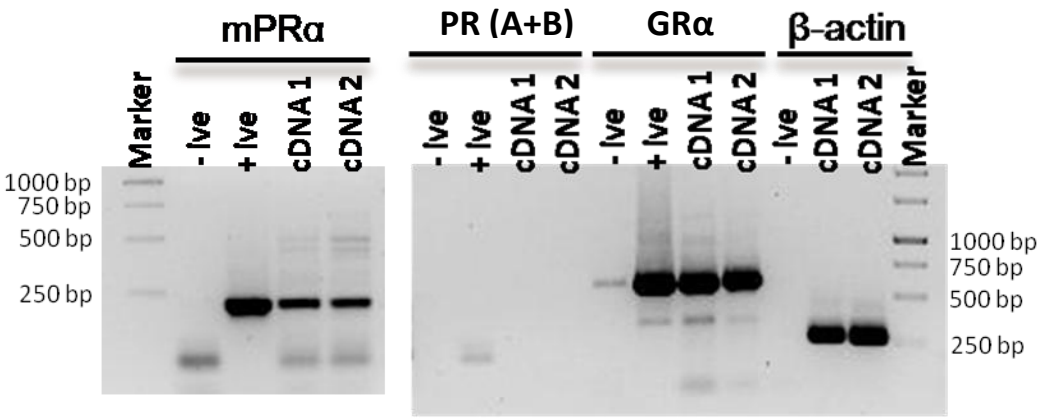


Figure 9.2: The mPRα and the GR, but not the PR, are endogenously expressed in the End-1 cell line. Endocervical cells (End-1were seeded into 6-well culture plates in KSF supplemented with 10% BPE, and left to grow to 70% confluency under basal conditions. Thereafter the cells were harvested and RNA extractions were performed, followed by a reverse transcriptase reaction to generate cDNA. Different biological repeats are annotated cDNA1 and cDNA2. cDNA was amplified using conventional PCR with gene-specific primer pairs (Addendum B). PCR products were generated over 35 PCR cycles, separated on a 2% agarose gel using electrophoreses, and visualised with ethidium bromide staining. Gel image shows mPRα and GR mRNA are basally expressed, while no PR mRNA can be found in the Endo-cervical cell line. β-actin used as an internal control. Signals are seen for the mPRα amplicon at 214 bp, the PR-B amplicon at 196 bp, the GR amplicon at 643 bp and the β-actin amplicon at 275 bp. Positive controls (+'ive) include the pcDNA3.1/mPRα, pMT-PR-B and pCMV-HA-hGR expression constructs. Negitive control (-'ive) defines the no template control.

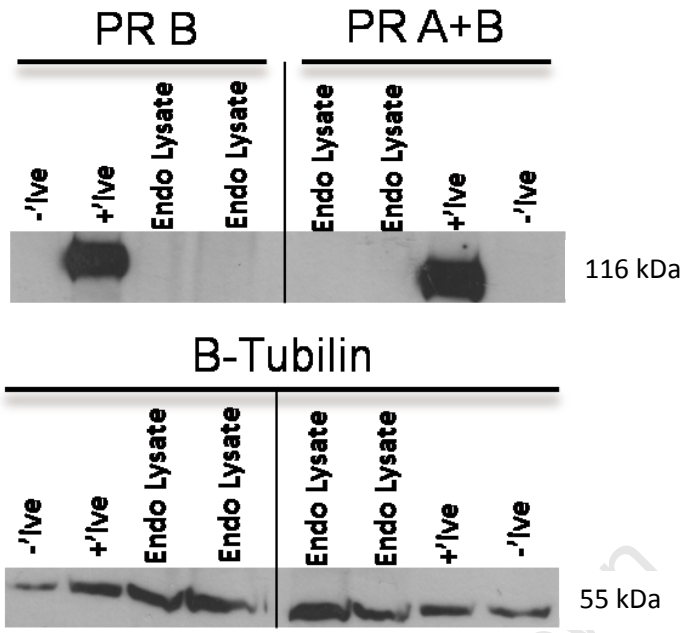


Figure 9.3: PR A + B protein is not detectable by Western blotting in the endo-cervical cell line. Endo-cervical cells were seeded into 6-well culture plates in KSF supplemented with 10% BPE and antibiotics and were left to grow to 70% confluency under basal conditions. Thereafter the cells were harvested in 100 µl 2x SDS sample application buffer. Equal amounts (10 µl) of Endo-cervical whole cell lysates were separated on a 8% SDS denaturing polyacrylamide gel, transferred to a nitrocellulose membrane and probed with PR-B-specific, PR(A+B)-specific and β-tubilin-specific (loading control) primary antibodies. Anti-mouse HRP conjugate secondary antibodies were used for detection of protein-specific primary antibodies. Signals were visualised with Amersham Chemiluminescence. PR-B and β-Tubilin proteins are observed at 116 kDa and 55 kDa respectively, while no signal is seen for PR-A (result not shown) at 81 kDa. Positive controls (+'ive) are COS-7 cells lysates transiently transfected with pMT-PR-B expression construct. Negitive control (-'ive) defines no template control.

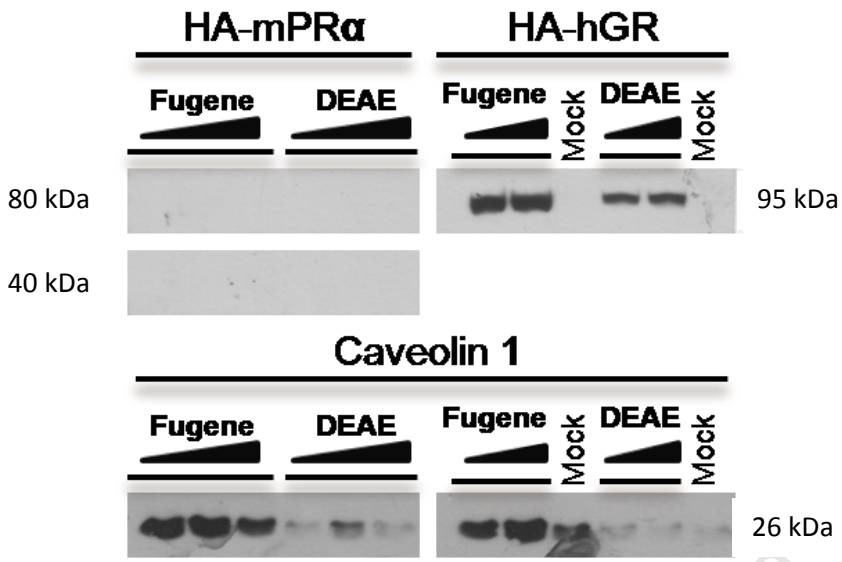


Figure 9.4: Transient expression of the HA-tagged human mPRα construct is not detected in the COS-7 cell line. COS-7 cells were seeded into 12-well culture plates in DMEM supplemented with 10% FCS. Twenty four hours after seeding, the cells were transiently transfected using Fugene or DEAE Dextran transfection protocols (Methods), with increasing amounts of either pHA/hmPRα (250 ng, 500 ng and 1 µg) or pCMV-HA-hGR (500 ng and 1 µg) and left to grow to 80% confluency under basal conditions. Thereafter the cells were harvested in 50 µl 2x SDS sample application buffer. Equal amounts (10 µl) of COS-7 whole cell lysates were separated on an 8% SDS denaturing polyacrylamide gel, transferred to a nitrocellulose membrane and probed with anti-HA and anti-Caveolin-1 (Loading control) primary antibodies. Anti-rabbit HRP conjugate secondary antibodies were used for detection of anti-HA primary antibodies, while anti-mouse HRP conjugate secondary antibodies were used for detection of anti-Caveolin-1 primary antibodies. Signals were visualised using Amersham Chemiluminescence. No expression of HA tagged hmPRα protein is detected at 40 kDa or 80 kDa. HA tagged GR is detected at 95 kDa. Caveolin-1 protein is at 26 kDa, and was used as a loading control, to assess total protein loaded. Mock defines the untransfected control.

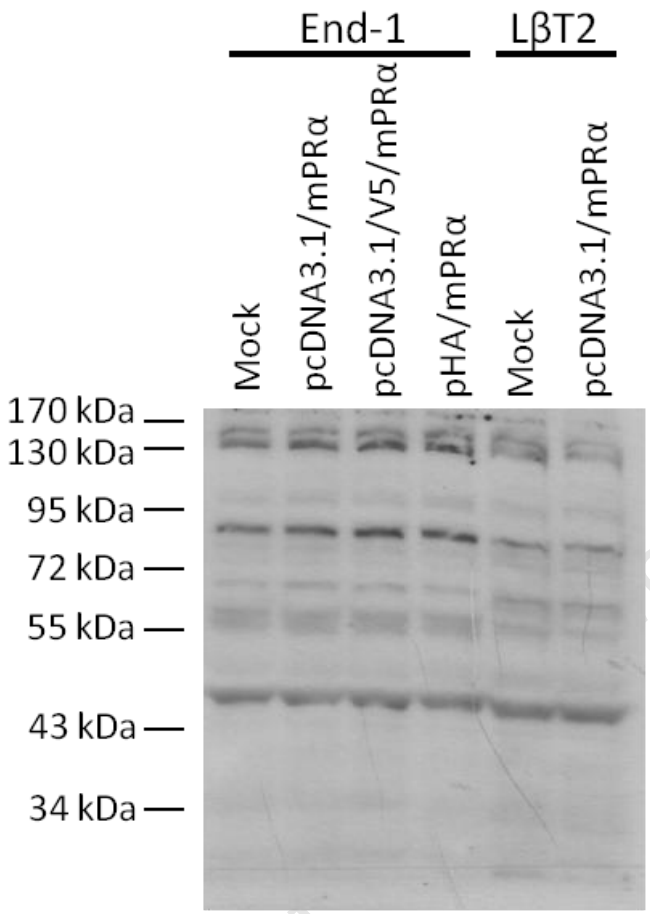


Figure 9.5: Transient expression of recombinant human mPRα protein is not detected in the LβT2 and End-1 cell lines. Endo-cervical cells were seeded into 12-well culture plates in KSF supplemented with 10% BPE. LβT2 cells were plated into 12-well plates in DMEM supplemented with 10% serum. After a 24 hour incubation period, the medium was replaced for both cell lines and incubated further for 24 hours. Thereafter 1 µg of pcDNA3.1/mPRα expression plasmid was transiently transfected and were left to grow to 80% confluency. Thereafter the cells were harvested in 50 µl 2x SDS sample application buffer. Equal amounts (10 µl) of End-1 and LβT2 whole cell lysates were separated on an 8% SDS denaturing polyacrylamide gel, transferred to a nitrocellulose membrane and probed with anti-mPRα primary antibodies. Anti-goat HRP conjugate secondary antibodies were used for detection of anti-mPRα primary antibodies. Signals were visualised using Amersham Chemiluminescence. mPRα exists as a 80 kDa homodimer or a 40 kDa monomer. Loading control showed sufficient protein loaded for each sample (data not shown). Mock defines the untransfected control.

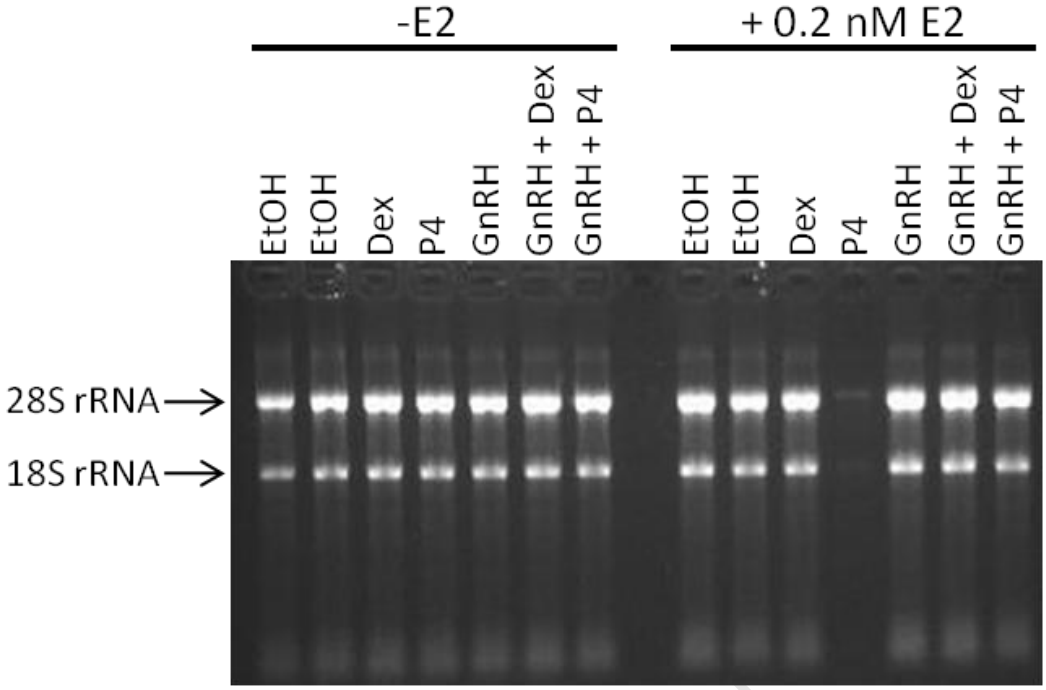


Figure 9.6: A representation of sample set RNA integrity for cDNA conversion and subsequent real-time PCR analysis using gene-specific primers. LβT2 cells were plated into 12-well plates in DMEM supplemented with 10% serum. After a 24 hours incubation period, the cells were treated with or without 0.2 nM E2 in phenol red-free media supplemented with 10% charcoal stripped serum. After 48 hours, the cells were treated with 100 nM vehicle (EtOH) or the appropriate ligands or combinations thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting and subsequent RNA extractions. RNA integrity was assessed by electrophoresis of RNA sample on a 1% denaturing RNA agarose gel. Image is a representation of sample RNA integrity for all biological repeats collected.

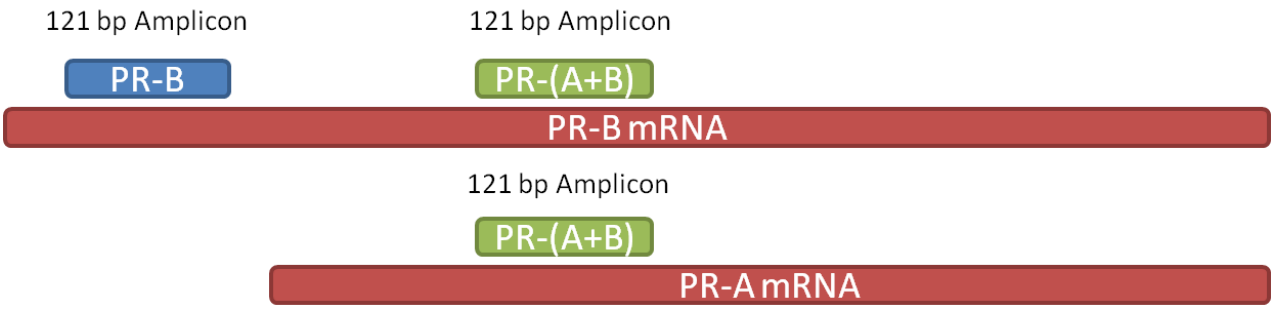


Figure 9.7: An illustration of where the PR-B and PR-(A+B) primer pairs anneal to the PR-A/B mRNA. Figure illustrates the regions to which PR-specific primers anneal to the PR-A or PR-B mRNA transcripts

Table 9.1: Summary of hmPRα overexpression study performed in the LβT2 and End-1 cell lines.

Cell line; Transfection & Harvesting Conditions			Observations			
Cell line	**Transfection Protocol**	**Harvesting Protocol**	**Endogenous mRNA expression of mPRα**	**Endogenous mPRα protein expression**	**Transfection Control (pCMV-HA-hGR)**	***Overexpression of mPRα protein**
End-1	DEAE Dextran	CREB/ HEPES	Yes	N.A.	Yes	No
LβT2	Fugene	HEPES	No	No	Yes	No
End-1	Fugene	CREB/ HEPES	Yes	N.A.	Yes	No
End-1	DEAE Dextran	5xSDS (50mM DTT)	Yes	No	Yes	No
LβT2	Fugene	5x SDS (50mM DTT)	No	No	Yes	No

Endo-cervical cells were seeded into 6-well culture plates in KSF supplemented with 10% BPE and antibiotics. LβT2 cells were seeded into 6-well culture plates in DMEM supplemented with 10% FCS and antibiotics. After 24 hours both cell lines were transiently transfected, using Fugene or DEAE Dextran transfection protocols, with 1 and 0.5 µg pcDNA3.1/hmPRα and pCMV-HA-hGR (transfection control) respectively, and left to grow to 70% confluency under basal conditions. Thereafter cells were harvested using harvesting protocols presented in methods section (Table 1). Equal amounts (10 µl) of whole cell lysates were separated on an 8% SDS denaturing polyacrylamide gel, transferred to a nitrocellulose membrane and probed with anti-hmPRα and anti-GR specific primary antibodies. Anti-rabbit HRP conjugate secondary antibodies were used for detection of GR-specific primary antibodies, while anti-goat HRP conjugate secondary antibodies were used for detection of mPRα primary antibodies. Mock transfections were used as untransfected (negative) controls. A summary of the observations during mPRα over-expression are summarized in table 9.1. Cell lines, tranfection protocols and harvesting protocols used are indicated. Observations show observations made during Western blot analysis.*Overexpression of mPRα protein is determined by measuring the differences between mock tranfected and mPRα transfected cells. If there is no difference in band intersity for mPRα signal, then no overexpression is seen. N.A. = No answer.

ADDENDUM B

PRIMER DESIGN

Table 10.1: Summery of the primers used in real-time and conventional PCR analysis.

Gene	Species	Primer Sequence	Strand	Product Size (bp)	Length	GC Content (%)	T_A (°C)	Reference	NCBI Hit
PR A+B	Mouse	GGTGGGCCTTCCTAACGAG	Fwd	121	19	63.2	60	Turgeon *et al.,* 2006	NM_008829.2
		GACCACATCAGGCTCAATGCT	Revs		21	54.2			
PR B	Mouse	GGTCCCCCTTGCTTGCA	Fwd	121	17	64.7	60	Turgeon *et al.,* 2006	NM_008829.2
		CAGGACCGAGGAAAAAGCAG	Revs		20	55			
ERα	Mouse	GTCTGGTCCTGCGAAGGCTGCAA	Fwd	235	23	60.9	60	Schreihofer *et al.,* 2000	NM_007956.4
		GCCTTCCAAGTCATCTCTCTGACG	Revs		24	54.2			
ERβ	Mouse	GCTGTGATGAACTACAGTGTTCCC	Fwd	267	24	50	60	Schreihofer *et al.,* 2000	NM_207707.1
		TGGACTAGTAAGTCATCTCTCTGACG	Revs		24	54.2			
GRα	Mouse	TGCTATGCTTTGCTCCTGATCTG	Fwd	299	23	47.8	52	Thackray *et al.,* 2006	NM_008173.3
		TGTCAGTTGATAAAACCGCTGCC	Revs		23	47.8			
AR	Mouse	GAGAACCCATTGGACTACG	Fwd	544	19	52.6	52	Thackray *et al.,* 2006	NM_013476.3
		TGAAGAAGACCTTGCAGC	Revs		18	50			
mPRα	Human	CGTTTCGGTCCACTGATCCCGG	Fwd	214	22	64	56	Jaravaza. (Hons Thesis)	NM_178422
		GCGAGAAGACCTTCGGCATGTAGATACG	Revs		28	54			
LHβ	Mouse	GGCCGCAGAGAATGAGTTCT	Fwd	83	20	55	60	Chen *et al.,* 2009	NM_008497.2
		CTCGGACCATGCTAGGACAGTAG	Revs		23	56.5			
GnRHR	Mouse	CCACAGTGGTGGCATCAGGCCTTC	Fwd	192	24	63	58	Kotitschke *et al.,* 2009 (PhD)	NM_010323.1
		TAGCGTTCTCAGCCGAGCTCTTGG	Revs		24	58			
GAPDH	Mouse	TTCACCACCATGGAGAAGGC	Fwd	263	20	55	58	Kotitschke *et al.,* 2009 (PhD)	NM_008084.2
		GGCATGGACTGTGGTCATCA	Revs		20	55			

Represented above is information regarding primer design and referencing for each gene-specific primer.

ADDENDUM C

PRIMER EFFICIENCY AND REAL-TIME PCR

11.1 Real-time PCR reproducibility

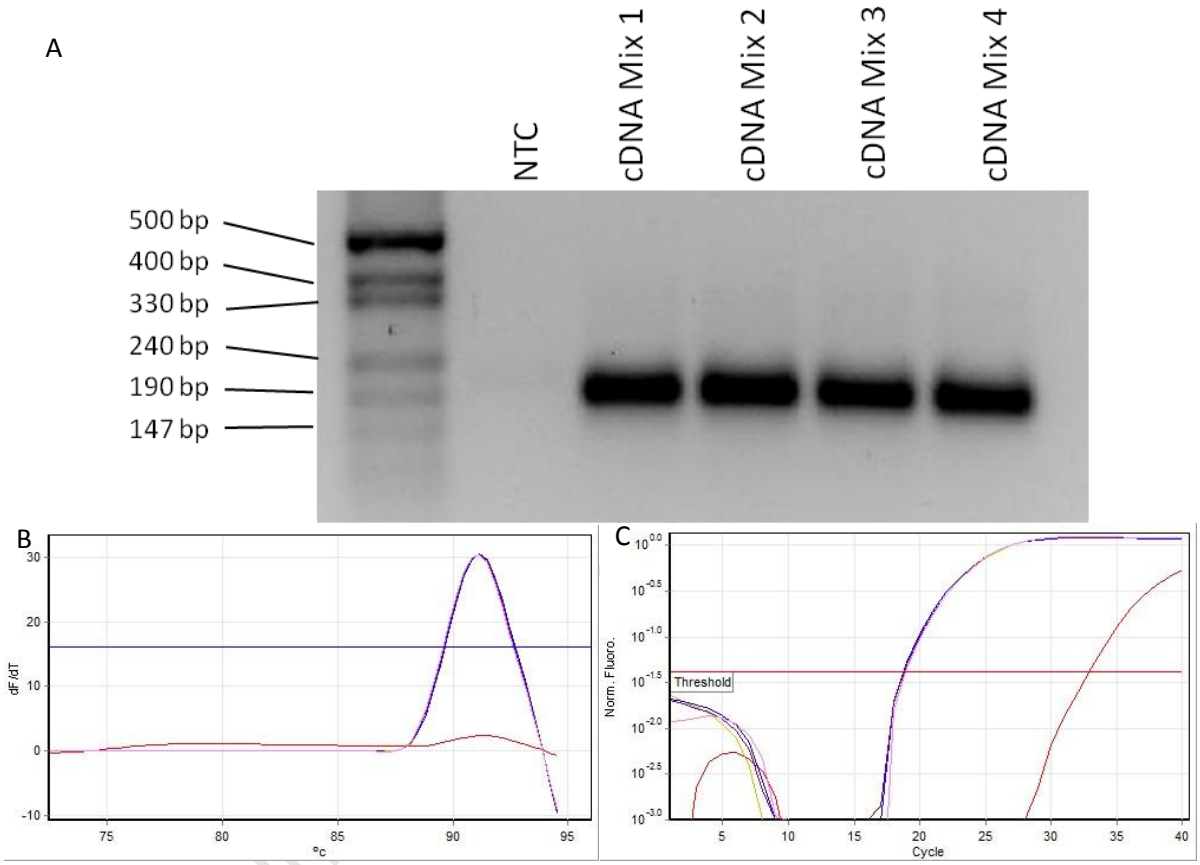


Figure 11.1 (A-C): Real-time PCR reproducibility using GnRHR primers on pooled LβT2 cDNA samples. LβT2 cells were plated in DMEM supplemented with 10% FCS. After 24 hours incubation, the cells were treated with or without 0.2 nM E2 in phenol red-free media supplemented with 10% charcoal stripped serum. After an additional 48 hours, the cells were treated with 100 nM vehicle (EtOH) or the appropriate ligands or combinations thereof. The cells were harvested, RNA was extracted, and cDNA was generated. Equal volumes (1 µl) of pooled endogenous cDNA (cDNA Mix #) were used to do 4x real time PCR reactions in paralle from the same cDNA pool to determine real-time reproducibility. Quantitative real-time PCR products were visualised on a 2% agarose gel using gel electrophoresis (A) and each product was confirmed using melting curve analysis (B). NTC defines the no template control. The GnRHR PCR product is seen at 192 bp. C_T values were calculated using

Roto Gene 6000 Corbrett™ software analysis (C). Melting points and C_T values are represented in Table 11.1.

Table 11.1: Melting temperatures and C_T values for GnRHR real-time PCR reproducibility.

Melt				Quantitation			
No.	**Colour**	**Name**	**Peak 1**	**No.**	**Colour**	**Name**	**C_T Value**
1		GnRHR NTC		**1**		GnRHR NTC	32.86
2		GnRHR Mix 1	91.2	**2**		GnRHR Mix 1	18.79
3		GnRHR Mix 2	91.2	**3**		GnRHR Mix 2	18.72
4		GnRHR Mix 3	91.2	**4**		GnRHR Mix 3	18.79
5		GnRHR Mix 4	91.0	**5**		GnRHR Mix 4	18.89

Table representing primer melt temperatures and C_T values determined from real-time PCR run. The table shows a high reproducibility of C_T values (averaging 18.8 cycles).

11.2 ERα primer efficiency

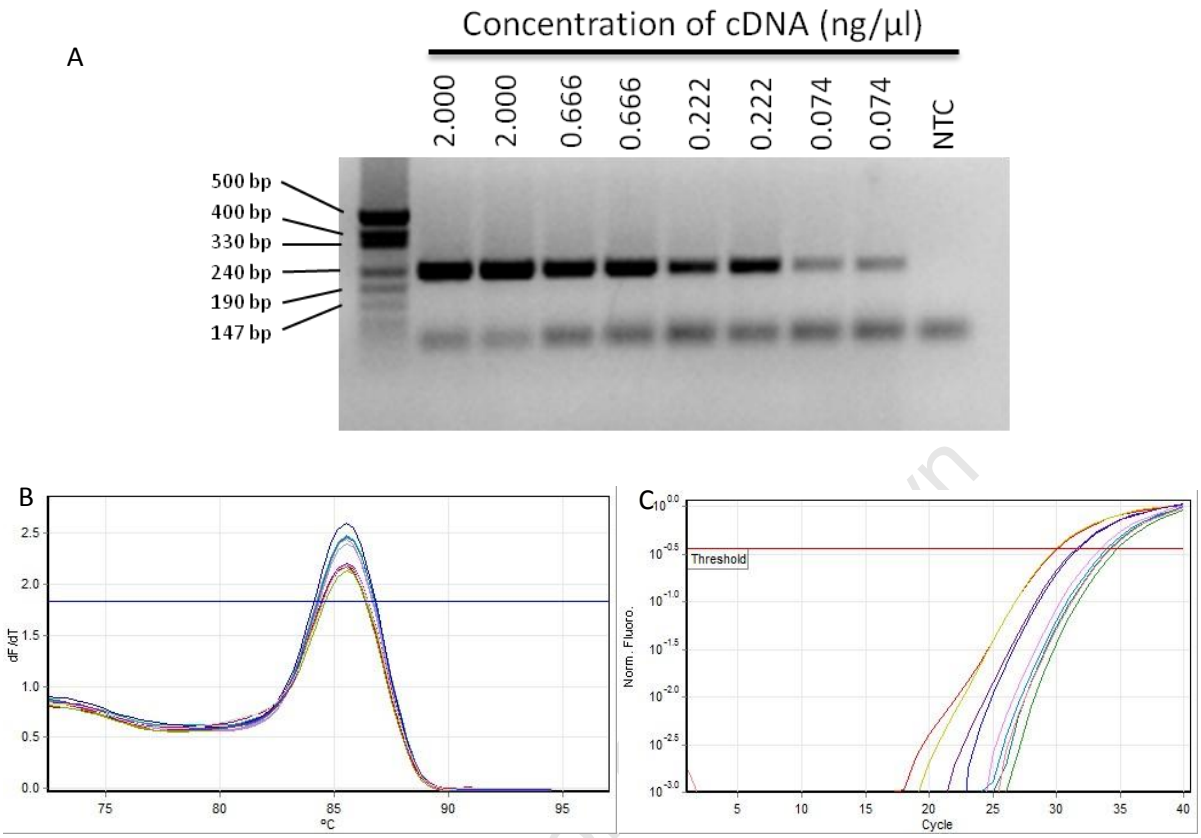
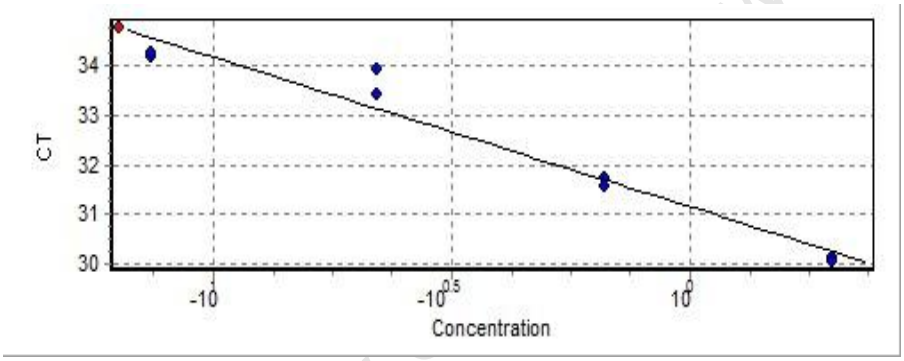


Figure 11.2.1 (A-C): Real-time PCR - ERα primer efficiency. LβT2 cells lysates were harvested, and subsequent RNA was extracted. cDNA was generated from RNA sample sets and pooled. A dilution series was made from pooled endogenous cDNA (concentration of pooled cDNA was 2 ng/µl). cDNA was diluted into 1:0, 1:3, 1:7 and 1:11 ratio's. Subsequent approximate concentrations were calculated (as shown on figure) and used to determine gene-specific primer efficiency. Quantitative real-time PCR products were visualised on a 2% agarose gel using gel electrophoresis (A) and each product was confirmed using melting curve analysis (B). NTC defines the no template control. C_T values were calculated using Roto Gene 6000 Corbrett™ software analysis (C) with C_T values represented in Table 11.2.1.

Table 11.2.1: Quantitation information for ERα primer efficiency.

No.	Colour	Name	C_T	Approx Conc (ng/µl)	Calc Conc (ng/µl)	% Var
1		ERa 1:0	30.13	2.000	2.175	8.8%
2		ERa 1:0	30.03	2.000	2.349	17.4%
3		ERa 1:3	31.74	.666	.639	4.0%
4		ERa 1:3	31.57	.666	.729	9.5%
5		ERa 1:7	33.44	.222	.177	20.2%
6		ERa 1:7	33.94	.222	.121	45.5%
7		ERa 1:11	34.19	.074	.100	35.3%
8		ERa 1:11	34.27	.074	.094	26.7%
9		ERa NTC	34.78		.064	

Table showing C_T values, relative concentration of cDNA and percentage variance for each cDNA dilution obtained from ERα primer efficiency real-time PCR.



Threshold	0.3669	B	31.1542
Left Threshold	1.000	R Value	0.97572
Standard Curve Imported	No	R^2 Value	0.95203
m	-3.03357	Reaction efficiency [E]	113.621

Figure 11.2.2: Standard curve C_T (cycle number) vs. concentration (ng/µl) to determine primer efficiency. C_T values collected from Table 11.2.1 above were plotted against their corresponding log concentrations, to generate a standard curve. The formula to calculate primer efficiency [E] percentage = (10^(-1/m)-1)*100, where m is the gradient of the standard curve. Gradient (m), R^2-value and primer efficiency are represented in table above. Efficiency of ERα primer pair was calculated to be 113%.

11.3 PR(A+B) primer efficiency

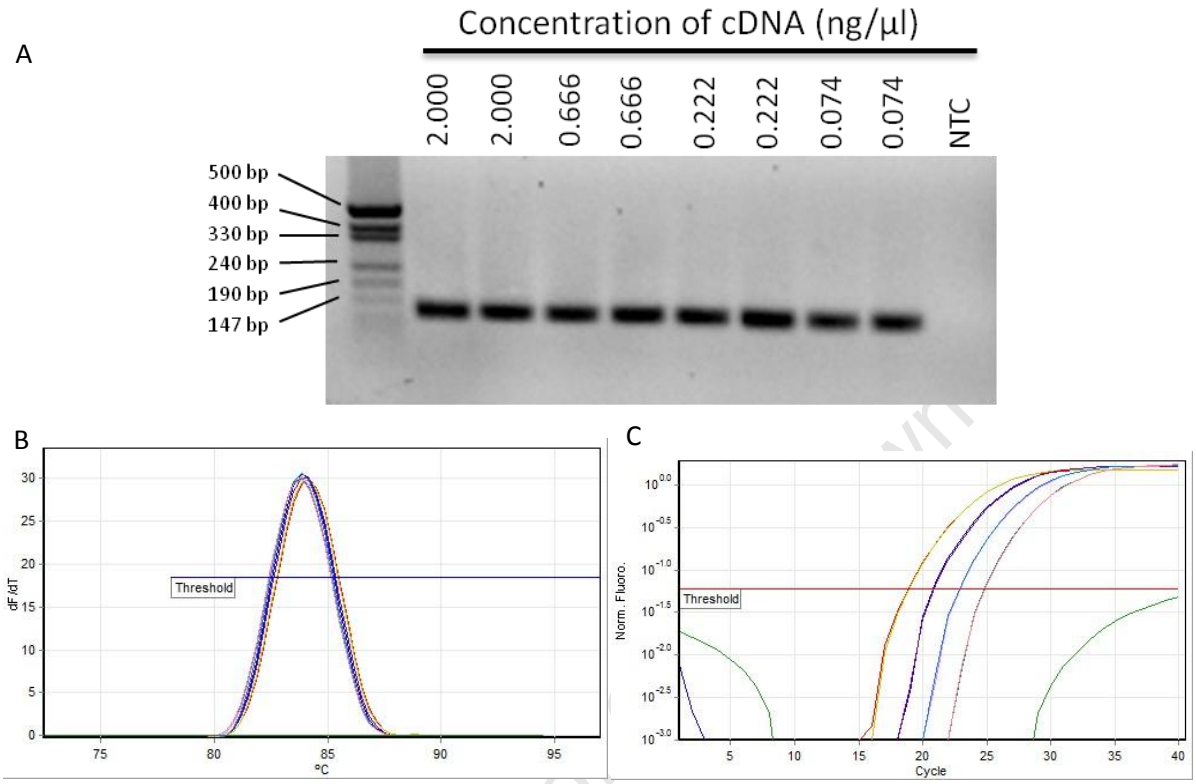
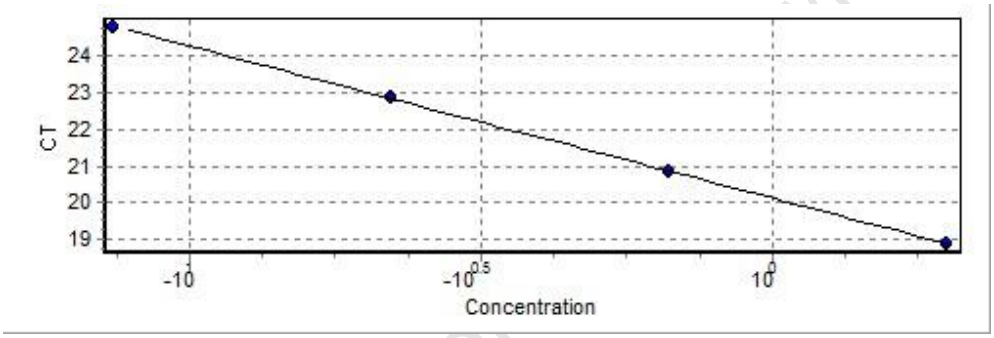


Figure 11.3.1 (A-C): Real-time PCR – PR (A+B) primer efficiency. LβT2 cells lysates were harvested, and subsequent RNA was extracted. cDNA was generated from RNA sample sets and pooled. A dilution series was made from pooled endogenous cDNA (concentration of pooled cDNA was 2 ng/µl). cDNA was diluted into 1:0, 1:3, 1:7 and 1:11 ratio's. Subsequent approximate concentrations were calculated (as shown on figure) and used to determine gene-specific primer efficiency. Quantitative real-time PCR products were visualised on a 2% agarose gel using gel electrophoresis (A) and each product was confirmed using melting curve analysis (B). NTC defines the no template control. C_T values were calculated using Roto Gene 6000 Corbrett^{TM} software analysis (C) with C_T values represented in Table 11.3.1.

Table 11.3.1: Quantitation information for PR(A+B) primer efficiency.

No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
A1		PR (A+B) 1:0	18.86	2.000	2.039	2.0%
A2		PR (A+B) 1:0	18.92	2.000	1.971	1.4%
A3		PR (A+B) 1:3	20.81	.666	.684	2.7%
A4		PR (A+B) 1:3	20.89	.666	.654	1.7%
A5		PR (A+B) 1:7	22.87	.222	.216	2.8%
A6		PR (A+B) 1:7	22.83	.222	.221	0.4%
A7		PR (A+B) 1:11	24.74	.074	.076	2.1%
A8		PR (A+B) 1:11	24.78	.074	.074	0.2%
B1		PR (A+B) NTC				

Table showing C_T values, concentration and percentage variance for each cDNA dilution gathered from PR-B primer efficiency real-time PCR.



Threshold	0.0603	B	20.13161
Left Threshold	1.000	R Value	0.99988
Standard Curve Imported	No	R^2 Value	0.99976
M	-4.11204	Reaction efficiency [E]	75.061

Figure 11.3.2: Standard curve C_T (cycle number) vs. concentration (ng/µl) to determine primer efficiency. C_T values collected from Table 11.3.1 above were plotted against their corresponding log concentrations, to generate a standard curve. The formula to calculate primer efficiency [E] percentage = (10^(-1/m)-1)*100, where m is the gradient of the standard curve. Gradient (m), R^2-value and primer efficiency are represented in table above. Efficiency of PR(A+B) primer pair was calculated to be 75%.

11.4 GRα primer efficiency

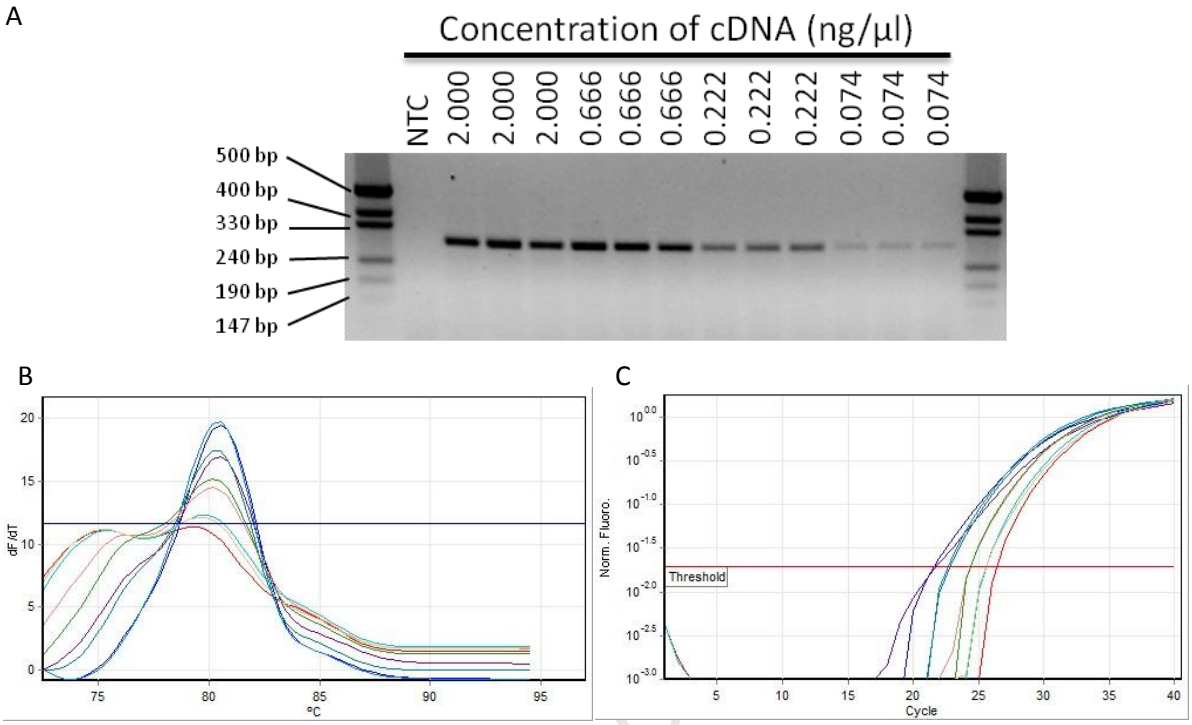
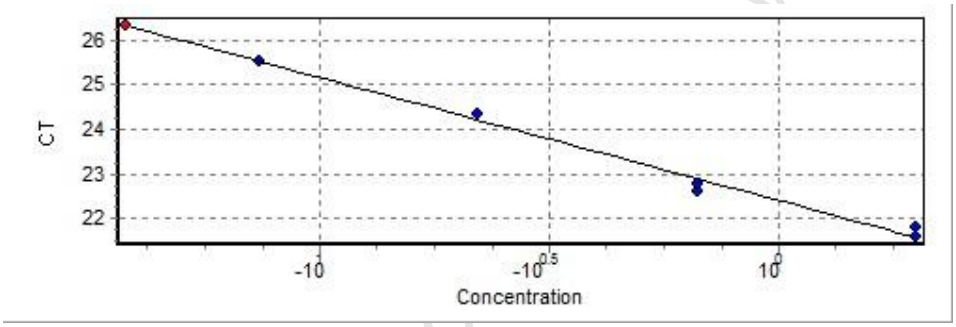


Figure 11.4.1 (A-C): Real-time PCR - GRα primer efficiency. LβT2 cells lysates were harvested, and subsequent RNA was extracted. cDNA was generated from RNA sample sets and pooled. A dilution series was made from pooled endogenous cDNA (concentration of pooled cDNA was 2 ng/µl). cDNA was diluted into 1:0, 1:3, 1:7 and 1:11 ratio's. Subsequent approximate concentrations were calculated (as shown on figure) and used to determine gene-specific primer efficiency. Quantitative real-time PCR products were visualised on a 2% agarose gel using gel electrophoresis (A) and each product was confirmed using melting curve analysis (B). NTC defines the no template control. C_T values were calculated using Roto Gene 6000 Corbrett™ software analysis (C) with C_T values represented in Table 11.4.1

Table 11.4.1: Quantitation information for GRα primer efficiency.

No.	Colour	Name	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
A1		GR NTC	26.32		.038	
A3		GR 1:0	21.59	2.000	1.988	0.6%
A4		GR 1:0	21.79	2.000	1.683	15.8%
A6		GR 1:3	22.60	.666	.849	27.5%
A7		GR 1:3	22.75	.666	.751	12.8%
A8		GR 1:7	24.32	.222	.202	9.1%
B1		GR 1:7	24.32	.222	.202	9.1%
B4		GR 1:11	25.52	.074	.074	0.2%
B5		GR 1:11	25.52	.074	.074	0.4%

Table showing C_T values, concentration and percentage variance for each cDNA dilution gathered from GRα primer efficiency real-time PCR.



Threshold	0.0196	B	22.40877
Left Threshold	1.000	R Value	0.995
Standard Curve Imported	No	R^2 Value	0.99003
M	-2.75592	Reaction efficiency [E]	130.598

Figure 11.4.2: Standard curve C_T (cycle number) vs. concentration (ng/µl) to determine primer efficiency. C_T values collected from Table 4.1 above were plotted against their corresponding log concentrations, to generate a standard curve. The formula to calculate primer efficiency [E] percentage = (10^(-1/m)-1)*100, where m is the gradient of the standard curve. Gradient (m), R^2-value and primer efficiency are represented in table above. Efficiency of GRα primer pair was calculated to be 130%.

11.5 GnRHR real-time PCR product

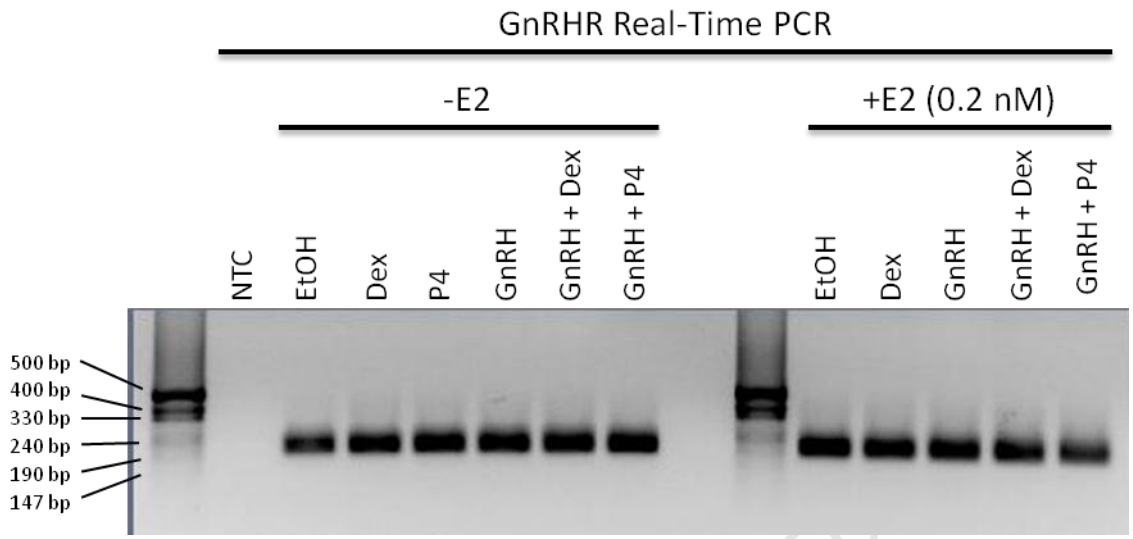


Figure 11.5.1: A representation of real-time PCR products using GnRHR primers. LβT2 cells were plated into 12-well culture plates in DMEM supplemented with 10% FCS. After 24 hours incubation, the cells were treated with (+E2) or without (-E2) 0.2 nM E2 in phenol red-free media supplemented with 10% charcoal stripped serum. After an additional 48 hours, the cells were treated with 100 nM vehicle (EtOH) or the appropriate ligands (Dex, P4, GnRH) or combinations thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting and subsequent RNA extractions. cDNA was generated for all mRNA sample sets. Quantitative real-time PCR using GnRHR-specific primers was performed. Amplicons were visualised using on a 2% agarose gel using gel electrophoresis, with melting curve analysis (data not shown) confirming GnRHR PCR product seen at 196 bp. NTC defines the no template control. C_T values were calculated using Roto Gene 6000 Corbrett™ software analysis (data not shown).

11.6 GAPDH real-time PCR product

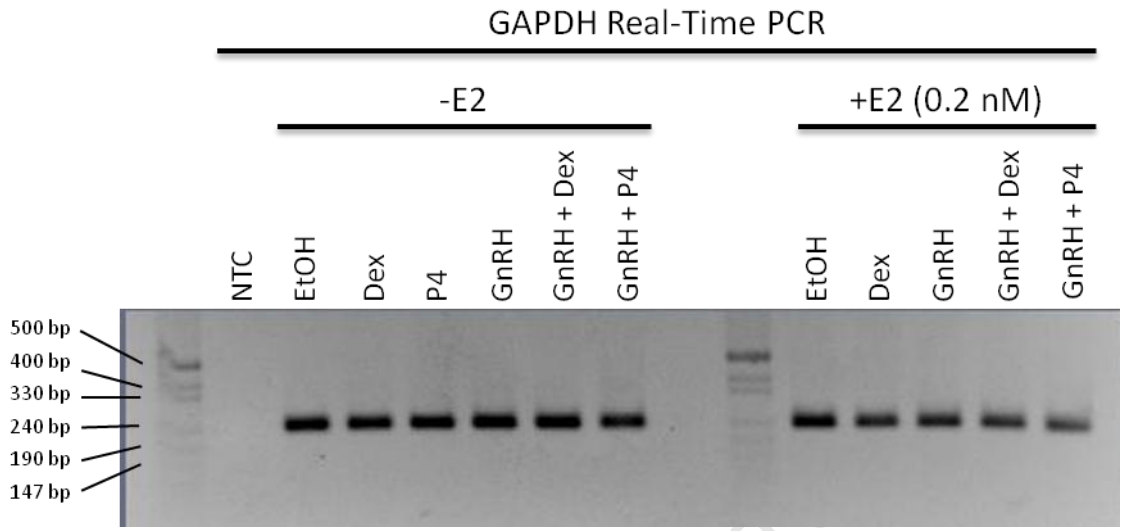


Figure 11.6.1: A representation of real-time PCR product using GAPDH primers. LβT2 cells were plated into 12-well culture plates in DMEM supplemented with 10% FCS. After 24 hours incubation, the cells were treated with (+E2) or without (-E2) 0.2 nM E2 in phenol red-free media supplemented (Zhao, 2008 #272)with 10% charcoal stripped serum. After an additional 48 hours, the cells were treated with 100 nM vehicle (EtOH) or the appropriate ligands (Dex, P4, GnRH) or combinations thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting and subsequent RNA extractions. cDNA was generated for all mRNA sample sets. Quantitative real-time PCR using GnRHR-specific primers was performed. Amplicons were visualised using on a 2% agarose gel using gel electrophoresis, with melting curve analysis (data not shown) confirming GnRHR PCR product seen at 263 bp. NTC defines the no template control C_T values were calculated using Roto Gene 6000 Corbrett™ software analysis (data not shown).

11.7 ERα real-time PCR product

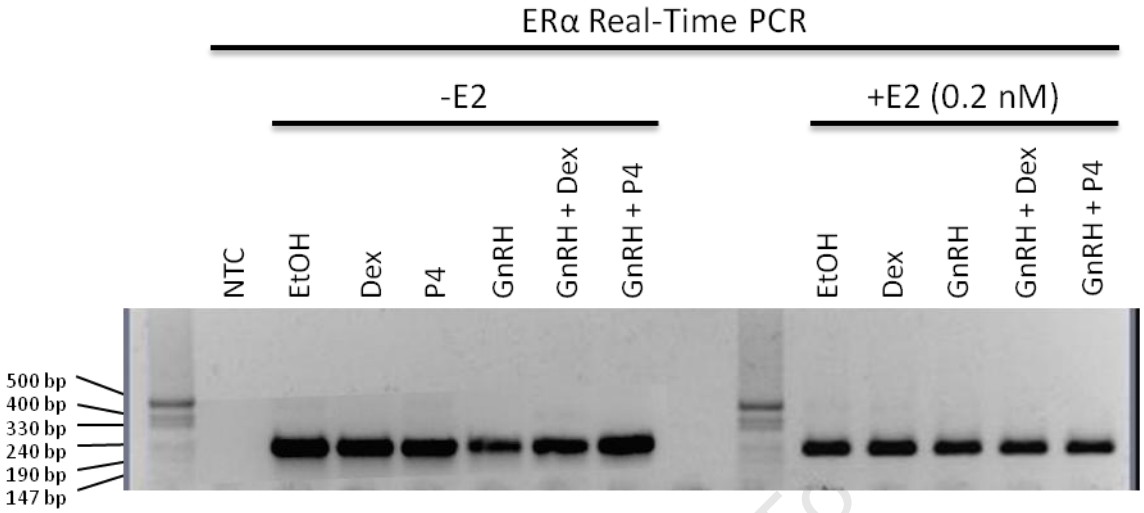


Figure 11.7.1: A representation of real-time PCR product using ERα primers. LβT2 cells were plated into 12-well culture plates in DMEM supplemented with 10% FCS. After 24 hours incubation, the cells were treated with (+E2) or without (-E2) 0.2 nM E2 in phenol red-free media supplemented with 10% charcoal stripped serum. After an additional 48 hours, the cells were treated with 100 nM vehicle (EtOH) or the appropriate ligands (Dex, P4, GnRH) or combinations thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting and subsequent RNA extractions. cDNA was generated for all mRNA sample sets. Quantitative real-time PCR using GnRHR-specific primers was performed. Amplicons were visualised using on a 2% agarose gel using gel electrophoresis, with melting curve analysis (data not shown) confirming GnRHR PCR product seen at 235 bp. NTC defines the no template control C_T values were calculated using Roto Gene 6000 Corbrett™ software analysis (data not shown).

11.8 PR(A+B) real-time PCR product

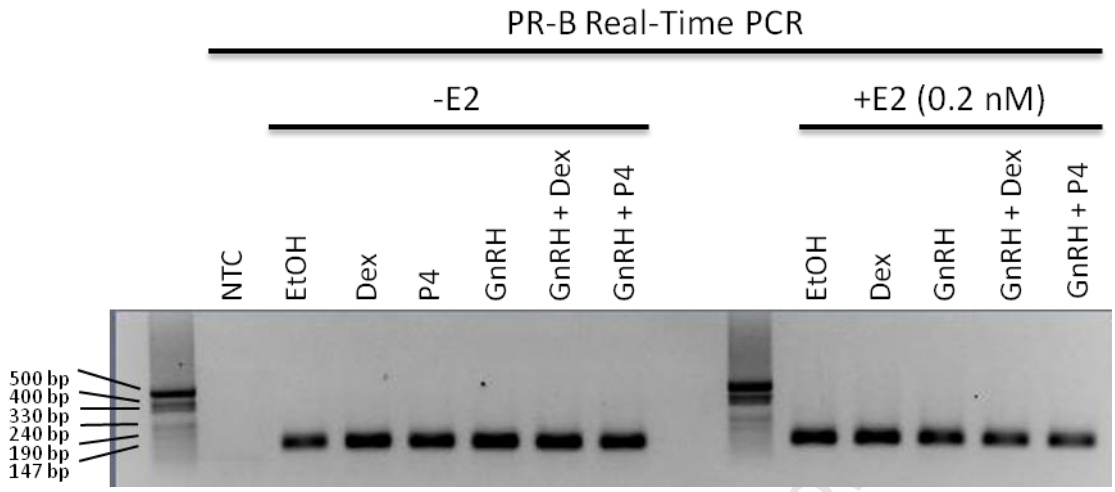


Figure 11.8.1: A representation of real-time PCR product using PR(A+B) primers. LβT2 cells were plated into 12-well culture plates in DMEM supplemented with 10% FCS. After 24 hours incubation, the cells were treated with (+E2) or without (-E2) 0.2 nM E2 in phenol red-free media supplemented with 10% charcoal stripped serum. After an additional 48 hours, the cells were treated with 100 nM vehicle (EtOH) or the appropriate ligands (Dex, P4, GnRH) or combinations thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting and subsequent RNA extractions. cDNA was generated for all mRNA sample sets. Quantitative real-time PCR using GnRHR-specific primers was performed. Amplicons were visualised using on a 2% agarose gel using gel electrophoresis, with melting curve analysis (data not shown) confirming GnRHR PCR product seen at 121 bp. NTC defines the no template control C_T values were calculated using Roto Gene 6000 Corbrett^{TM} software analysis (data not shown).

11.9 GRα real-time PCR product

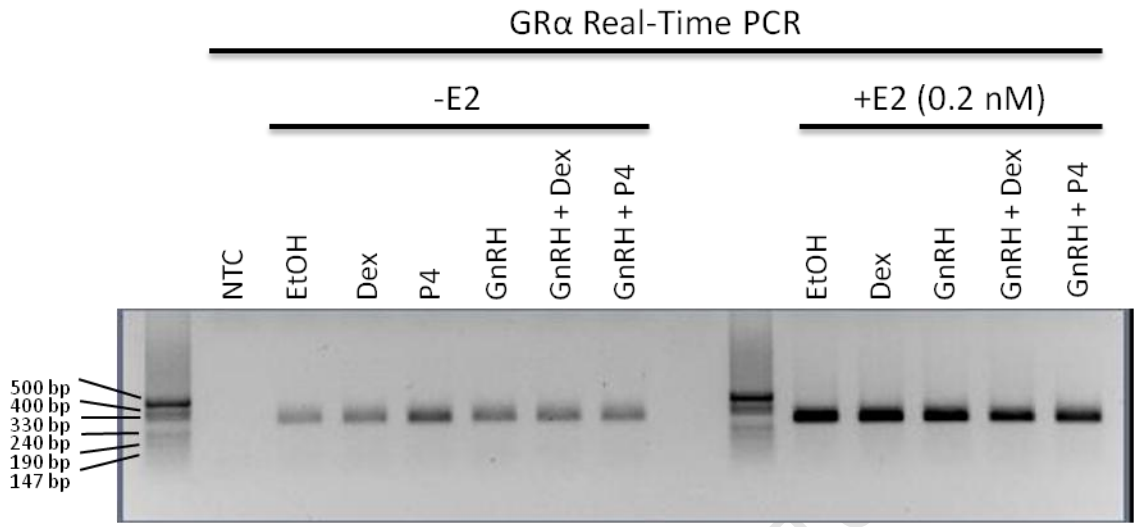


Figure 11.9.1: A representation of real-time PCR product using GRα primers. LβT2 cells were plated into 12-well culture plates in DMEM supplemented with 10% FCS. After 24 hours incubation, the cells were treated with (+E2) or without (-E2) 0.2 nM E2 in phenol red-free media supplemented with 10% charcoal stripped serum. After an additional 48 hours, the cells were treated with 100 nM vehicle (EtOH) or the appropriate ligands (Dex, P4, GnRH) or combinations thereof in phenol red-free, serum-free media. The cells were stimulated for 8 hours prior to harvesting and subsequent RNA extractions. cDNA was generated for all mRNA sample sets. Quantitative real-time PCR using GnRHR-specific primers was performed. Amplicons were visualised using on a 2% agarose gel using gel electrophoresis, with melting curve analysis (data not shown) confirming GnRHR PCR product seen at 299 bp. NTC defines the no template control. C_T values were calculated using Roto Gene 6000 Corbrett™ software analysis (data not shown).

ADDENDUM D

PLASMID MAPS

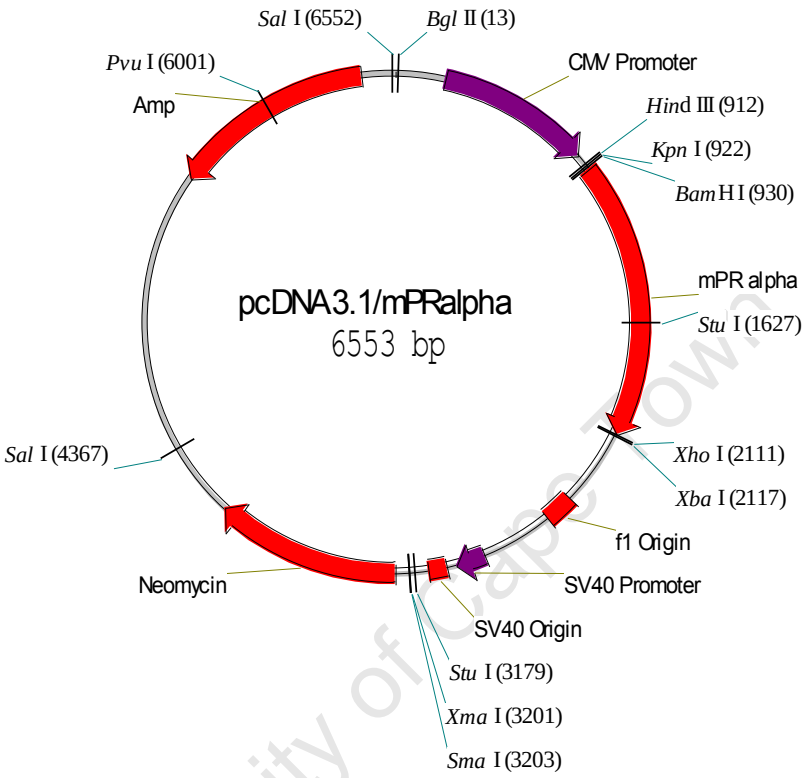


Figure 12.1: Plasmid map of pcDNA3.1/mPRα. The pcDNA3.1/V5/mPRα expression construct was a kind gift from Dr. B. Gellersen (Endokrinologikum Hamburg, Germany), however, plasmid maps were not provided. The human mPRα cDNA insert was sub-cloned into the pcDNA3.1 expression vector. The expression construct was sequenced and digested using appropriate restriction enzymes and a detailed plasmid map was constructed by the candidate using Vector NTI software.

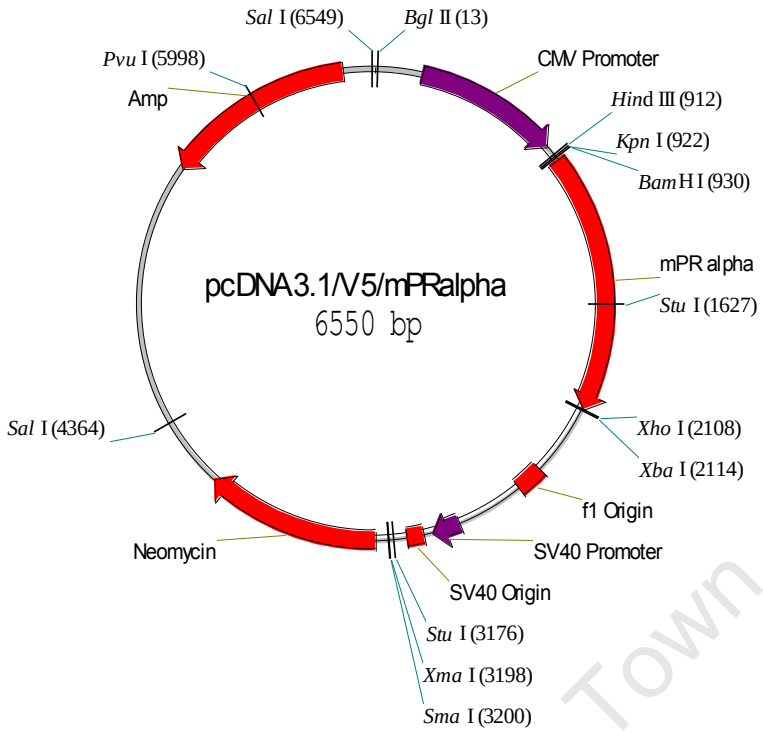


Figure 12.2: Plasmid map of pcDNA3.1/V5/mPRα. The pcDNA3.1/V5/mPRα expression construct was a kind gift from Dr. B. Gellersen (Endokrinologikum Hamburg, Germany), however, plasmid maps were not provided. The human mPRα cDNA insert was sub-cloned into the pcDNA3.1 expression vector with a 3' V5-His tag. The expression construct was sequenced and digested using appropriate restriction enzymes and a detailed plasmid map was constructed by the candidate using Vector NTI software.

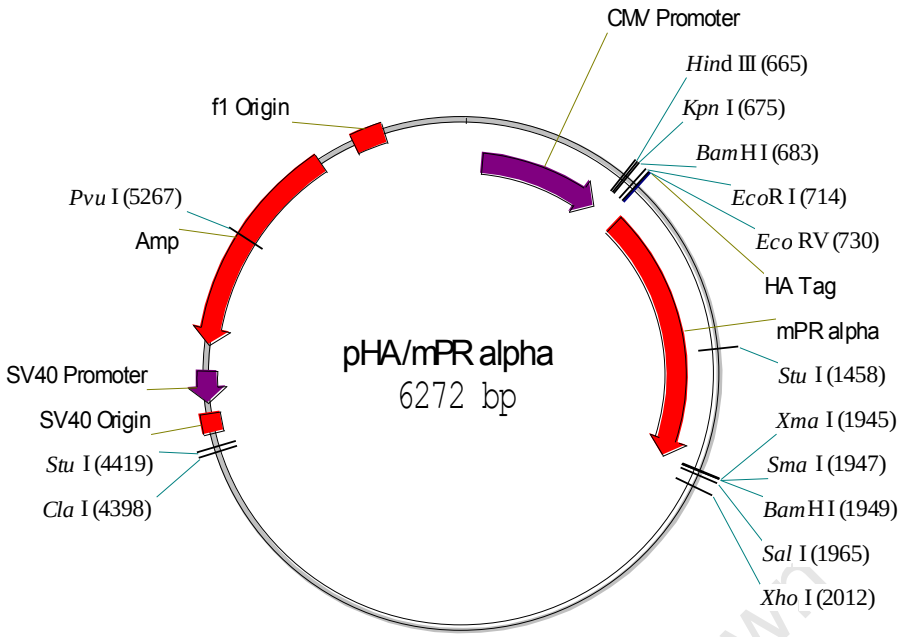


Figure 12.3: Plasmid map of pHA/mPRα. The pcDNA3.1/V5/mPRα expression construct was a kind gift from Dr. B. Gellersen (Endokrinologikum Hamburg, Germany), however, plasmid maps were not provided. The human mPRα cDNA insert was sub-cloned into a modified pDISPLAY expression vector, from which Igκ signal peptide and TMD have been deleted. The expression construct was sequenced and digested using appropriate restriction enzymes and a detailed plasmid map was constructed by the candidate using Vector NTI software.

ADDENDUM E

CALCULATIONS FOR STATISTICAL ANALYSIS

All statistical analysis for this study was performed using Prism 5, GraphPad Software. Statistical analysis included one-way ANOVA, with Dunnett post test, two-way ANOVA with Bonferroni post test and a T-test. It must be noted that calculated p values were not displayed due to limitations of the software. Instead a p value summery is shown, to determine statistical significance.

Table 13.1: Statistical analysis of TAT-GRE-luc expression in response to hormone conditions represented in figure 3.1

Non-parametric one-way ANOVA (Figure 3.1)				
Dunnett's post-test	Mean Diff.	95% CI of diff	Significant? P < 0.05?	Summary
EtOH (1) vs Dex (2)	*-2.66*	*-8.457 to 3.138*	*No*	*ns*
EtOH (1) vs P4 (3)	*0.2433*	*-5.554 to 6.041*	*No*	*ns*
EtOH (1) vs GnRH (4)	*-0.457*	*-6.255 to 5.341*	*No*	*ns*
EtOH (1) vs GnRH + Dex (5)	*-15.24*	*-21.04 to -9.442*	*Yes*	*****
EtOH (1) vs GnRH + P4 (6)	*-0.2908*	*-6.088 to 5.507*	*No*	*ns*

Table 13.2: Statistical analysis of endogenous *GnRHR* mRNA expression in response to hormone conditions represented in figure 3.2

Non-parametric two-way ANOVA (Figure 3.2)				
Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	*2.05*	*0.9491*	*ns*	*No*
Treatment	*41.95*	*0.0035*	****	*Yes*
Priming	*0.13*	*0.7934*	*ns*	*No*

Bonferroni post-tests (Figure 3.2)						
EtOH vs Dex						
Priming	EtOH	Dex	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*4.27*	*3.27*	*-3.650 to 10.19*	*P > 0.05*	*ns*
+0.2 nM E2	*1.34*	*6.052*	*4.712*	*-3.278 to 12.70*	*P > 0.05*	*ns*
EtOH vs P4						
Priming	EtOH	P4	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*1.36*	*0.3602*	*-6.560 to 7.280*	*P > 0.05*	*ns*
+0.2 nM E2	*1.34*	*2.259*	*0.9188*	*-10.38 to 12.22*	*P > 0.05*	*ns*
EtOH vs GnRH						
Priming	EtOH	GnRH	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*3.065*	*2.065*	*-4.855 to 8.985*	*P > 0.05*	*ns*
+0.2 nM E2	*1.34*	*3.205*	*1.865*	*-6.125 to 9.855*	*P > 0.05*	*ns*
EtOH vs GnRH + Dex						

Priming	EtOH	GnRH + Dex	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*9.457*	*8.457*	*1.537 to 15.38*	*P<0.01*	**
+0.2 nM E2	*1.34*	*7.666*	*6.326*	*-1.664 to 14.32*	*P < 0.05*	*
EtOH vs GnRH + P4						
Priming	EtOH	GnRH + P4	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*2.664*	*1.664*	*-5.256 to 8.584*	*P > 0.05*	*ns*
+0.2 nM E2	*1.34*	*3.028*	*1.688*	*-6.302 to 9.678*	*P > 0.05*	*ns*

Table 13.3: Statistical analysis of ERE-luc reporter expression in response to agonist treatment represented in figure 4.3

T-test (Figure 4.3)						
Receptor	EtOH	Agonist	Difference	95% CI of diff.	P value	Summary
ER	*1*	*1.212*	*0.2122*	*-1.378 to 1.803*	*P > 0.05*	*ns*

Table 13.4: Statistical analysis of endogenous *ERα* mRNA expression in response to hormone conditions represented in figure 4.4

Non-parametric two-way ANOVA (Figure 4.4)				
Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	*4.48*	*0.2511*	*ns*	*No*
Treatment	*77.17*	*P<0.0001*	***	*Yes*
E2 Priming	*2.19*	*0.0741*	*ns*	*No*

Bonferroni post-tests						
EtOH vs Dex						
E2 Priming	EtOH	Dex	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.8818*	*-0.1182*	*-0.5869 to 0.3505*	*P > 0.05*	*ns*
+0.2 nM E2	*1.035*	*1.416*	*0.3809*	*-0.1603 to 0.9222*	*P > 0.05*	*ns*
EtOH vs P4						
E2 Priming	EtOH	P4	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.5442*	*-0.4558*	*-0.9246 to 0.01290*	*P < 0.05*	*
+0.2 nM E2	*1.035*	*0.6566*	*-0.378*	*-1.143 to 0.3874*	*P > 0.05*	*ns*
EtOH vs GnRH						
E2 Priming	EtOH	GnRH	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.2339*	*-0.7661*	*-1.235 to -0.2974*	*P<0.001*	***
+0.2 nM E2	*1.035*	*0.2464*	*-0.7882*	*-1.329 to -0.2470*	*P<0.001*	***
EtOH vs GnRH + Dex						
E2 Priming	EtOH	GnRH + Dex	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.2185*	*-0.7815*	*-1.250 to -0.3127*	*P<0.001*	***
+0.2 nM E2	*1.035*	*0.3041*	*-0.7305*	*-1.272 to -0.1893*	*P<0.001*	***
EtOH vs GnRH + P4						
E2 Priming	EtOH	GnRH + P4	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.2322*	*-0.7678*	*-1.237 to -0.2991*	*P<0.001*	***

+0.2 nM E2	*1.035*	*0.2775*	*-0.7571*	*-1.298 to -0.2159*	*P<0.001*	*****

Table 13.5: Statistical analysis of ERE-luc expression in response to 100 nM GnRH priming represented in figure 4.5

Non-parametric two-way ANOVA (Figure 4.5)				
Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	*0.09*	*0.8665*	*ns*	*No*
Agonist	*4.22*	*0.2765*	*ns*	*No*
GnRH Priming	*68.93*	*0.002*	****	*Yes*

Bonferroni post-tests						
EtOH vs agonist (E2)						
GnRH Priming	EtOH	E2	Difference	95% CI of diff.	P value	Summary
-100nM GnRH	*1*	*0.6368*	*-0.3632*	*-1.499 to 0.7724*	*P > 0.05*	*ns*
+100nM GnRH	*2.232*	*1.962*	*-0.2697*	*-1.285 to 0.7460*	*P > 0.05*	*ns*

Table 13.6: Statistical analysis of PRE-luc expression in response to agonist treatment represented in figure 5.3

T-test (Figure 5.3)						
Receptor	EtOH	Agonist	Difference	95% CI of diff.	P value	Summary
PR	*1*	*1.231*	*0.2315*	*-1.359 to 1.822*	*P > 0.05*	*ns*

Table 13.7: Statistical analysis of endogenous *PR (A+B)* mRNA expression in response to hormone conditions represented in figure 5.4

Non-parametric two-way ANOVA (Figure 5.4)				
Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	*11.21*	*0.4309*	*ns*	*No*
Treatment	*25.44*	*0.0774*	*ns*	*No*
E2 Priming	*1.32*	*0.4468*	*ns*	*No*

Bonferroni post-tests						
EtOH vs Dex						
E2 Priming	EtOH	Dex	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.8783*	*-0.1217*	*-1.415 to 1.171*	*P > 0.05*	*ns*
+0.2 nM E2	*1.09*	*0.962*	*-0.1277*	*-1.956 to 1.701*	*P > 0.05*	*ns*
EtOH vs P4						

E2 Priming	EtOH	P4	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.8873*	*-0.1127*	*-1.406 to 1.180*	*P > 0.05*	*ns*
+0.2 nM E2	*1.09*	*0.8184*	*-0.2713*	*-2.511 to 1.968*	*P > 0.05*	*ns*
EtOH vs GnRH						
E2 Priming	EtOH	GnRH	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*2.118*	*1.118*	*-0.1756 to 2.411*	*P < 0.05*	***
+0.2 nM E2	*1.09*	*1.434*	*0.3446*	*-1.484 to 2.173*	*P > 0.05*	*ns*
EtOH vs GnRH + Dex						
E2 Priming	EtOH	GnRH + Dex	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*1.99*	*0.9895*	*-0.3035 to 2.283*	*P > 0.05*	*ns*
+0.2 nM E2	*1.09*	*1.092*	*0.002326*	*-1.826 to 1.831*	*P > 0.05*	*ns*
EtOH vs GnRH + P4						
E2 Priming	EtOH	GnRH + P4	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*1.45*	*0.4504*	*-0.8427 to 1.744*	*P > 0.05*	*ns*
+0.2 nM E2	*1.09*	*1.907*	*0.8173*	*-1.011 to 2.646*	*P > 0.05*	*ns*

Table 13.8: Statistical analysis of PRE-luc expression in response to 100 nM GnRH priming represented in figure 5.5

Non-parametric two-way ANOVA (Figure 5.5)				
Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	*7.68*	*0.045*	*	*Yes*
Agonist	*0.31*	*0.6779*	*ns*	*No*
GnRH Priming	*35.56*	*P<0.0001*	***	*Yes*

Bonferroni post-tests						
EtOH vs agonist (R5020)						
GnRH Priming	EtOH	R 5 020	Difference	95% CI of diff.	P value	Summary
-100nM GnRH	*1*	*0.791*	*-0.209*	*-0.4866 to 0.06851*	*P > 0.05*	*ns*
+100nM GnRH	*1.201*	*1.34*	*0.1391*	*-0.1385 to 0.4166*	*P > 0.05*	*ns*

Table 13.9: Statistical analysis of PRE-luc expression in response to 0.2 nM E2 priming represented in figure 5.5

Non-parametric two-way ANOVA (Figure 5.5)				
Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	*0.75*	*0.4897*	*ns*	*No*
Agonist	*1.37*	*0.3519*	*ns*	*No*
E2 Priming	*67.74*	*P<0.0001*	*****	*Yes*

Bonferroni post-tests						
EtOH vs agonist (R5020)						
E2 Priming	EtOH	R5020	Difference	95% CI of diff.	P value	Summary
-0.2nM E2	*1*	*0.9761*	*-0.02385*	*-0.3515 to 0.3038*	*P > 0.05*	*ns*
+0.2nM E2	*1.708*	*1.55*	*-0.1585*	*-0.4861 to 0.1692*	*P > 0.05*	*ns*

Table 13.10: Statistical analysis of GRE-luc expression in response to agonist treatment represented in figure 6.3

T-test (Figure 6.3)						
Receptor	EtOH	Agonist	Difference	95% CI of diff.	P value	Summary
GR	*1*	*4.963*	*3.963*	*2.372 to 5.554*	*P<0.001*	*****

Table 13.11: Statistical analysis of endogenous *GRα* mRNA expression in response to hormone conditions represented in figure 6.4

Non-parametric two-way ANOVA (Figure 6.4)				
Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	*8.88*	*0.4564*	*ns*	*No*
Treatment	*42.64*	*0.0054*	****	*Yes*
E2 Priming	*4.6*	*0.1274*	*ns*	*No*

Bonferroni post-tests						
EtOH vs Dex						
E2 Priming	EtOH	Dex	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.49*	*-0.51*	*-0.9780 to -0.04206*	*P<0.01*	****
+0.2 nM E2	*0.9277*	*0.9174*	*-0.01037*	*-0.6722 to 0.6514*	*P > 0.05*	*ns*
EtOH vs P4						
E2 Priming	EtOH	P4	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.5378*	*-0.4622*	*-0.9302 to 0.005775*	*P < 0.05*	***
+0.2 nM E2	*0.9277*	*0.5822*	*-0.3455*	*-1.156 to 0.4650*	*P > 0.05*	*ns*

EtOH vs GnRH						
E2 Priming	EtOH	GnRH	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.5056*	*-0.4944*	*-0.9999 to 0.01106*	*P < 0.05*	*
+0.2 nM E2	*0.9277*	*0.498*	*-0.4297*	*-1.092 to 0.2321*	*P > 0.05*	*ns*
EtOH vs GnRH + Dex						
E2 Priming	EtOH	GnRH + Dex	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.3115*	*-0.6885*	*-1.194 to -0.1830*	*P<0.001*	***
+0.2 nM E2	*0.9277*	*0.4467*	*-0.481*	*-1.143 to 0.1808*	*P > 0.05*	*ns*
EtOH vs GnRH + P4						
E2 Priming	EtOH	GnRH + P4	Difference	95% CI of diff.	P value	Summary
-E2	*1*	*0.4604*	*-0.5396*	*-1.045 to -0.03415*	*P<0.01*	**
+0.2 nM E2	*0.9277*	*0.6981*	*-0.2297*	*-0.8915 to 0.4322*	*P > 0.05*	*ns*

ADDENDUM F

BUFFERS AND SOLUTIONS

14.1 Growth medium for plasmid preparation

SOC medium

2% (w/v) tryptone

0.5% (w/v) yeast extract

0.05% (w/v) NaCl

2.5 mM KCl

10 mM MgCl2

20 mM glucose

LB medium

1% (w/v) tryptone

0.5% yeast extract

1% NaCl

containing 50 µg/mL ampicillin

for LB-agar plates add 1,5% agar

14.2 RNA isolation

DEPC-treated H_2O

1 ml diethyl pyrocarbonate (DEPC) in 1 L dH_2O (1:1000 dilution)

Incubate 2 h at 37°C, autoclave twice to inactivate DEPC

10X Morpholinopropanesulfonic acid (MOPS) buffer

0.2 M MOPS

0,05 M sodium acetate

0,01M EDTA, pH 8.0, adjust pH to 7.0 with 10 M NaOH

Denaturing formaldehyde gel mix (1% 100 ml)

Dissolve 1 g agarose in 70 ml DEPC-treated H2O and bring to boil. Add 10 ml 10X MOPS buffer and 20 ml formaldehyde in fume hood. Mix well and pour.

RNA sample loading buffer

1,8 ml DEPC H2O

0,8 ml Bromophenol blue solution (saturated)

1 ml glycerol (100%)

1.5 ml 10x MOPS

2,6 ml 12.3 M formaldehyde

7,3 ml formamide

Add 2,5 µl 10 mg/ml ethidium bromide per 1 ml RNA sample loading buffer just before use.

RNA electrophoresis buffer (500 ml)

50 ml 1x MOPS

14 ml formaldehyde

436 ml DEPC-treated water

14.3 Western blot analysis

5X SDS sample buffer

100 mM Tris-HCL, pH 6.8

5% (w/v) SDS

20% (v/v) glycerol

2% (v/v) ß-mercaptoethanol

0.1% (w/v) bromophenol blue

Running buffer

25 mM Tris-HCL, pH 7.5

250 mM glycine

0,1% SDS (w/v)

Transfer buffer

25 mM Tris, pH 7.5

200 mM glycine

10% (v/v) methanol

Tris buffered saline (TBS)

50 mM Tris, pH 7.5

150 mM NaCl

TBS-Tween (TBS-T)

50 mM Tris, pH 7.5

150 mM NaCl

0.1% (v/v) Tween

Stripping buffer

100 mM ß-mercaptoethanol

2% (w/v) SDS

62.5 mM Tris-Cl, pH 6.8

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